# CHARACTERIZATION OF THE HUMAN INFLAMMATORY RESPONSE TO *GARDNERELLA VAGINALIS*

by

Eric Vick

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Molecular Bioscience

Middle Tennessee State University

December 2014

Dissertation committee:

Dr. Anthony L. Farone, Chair

Dr. Mary B. Farone

Dr. Matthew Elrod-Erickson

Dr. Rebecca L. Seipelt-Thiemann

Dr. Paul C. Kline

I dedicate this dissertation to my grandfather, Dr. R. C. Henderson, who was the original inspiration of my education, and the man who fostered my curiosity. I additionally dedicate this dissertation to my family, who has provided me with the support that has allowed me to reach this point. I would have never made it to this point without each of you.

## ACKNOWLEDGEMENTS

"We are at the very beginning of time for the human race. It is not unreasonable that we grapple with problems. But there are tens of thousands of years in the future. Our responsibility is to do what we can, learn what we can, improve the solutions, and pass them on. - Richard Feynman"

The phrase group effort does little justice to completion of this document. We have worked tirelessly to support and aid each other and I am thankful beyond clichés for the help each one you has given me.

First, I would like to acknowledge my advisors, Dr. Anthony L. Farone and Dr. Mary B. Farone for their inspiration, vigilance, patience, and advice over the years. They took me in when I needed a lab, treated me as family, and are the pillars of this work. Without them none of this would have been possible. I would also like to thank the other members of my committee: Dr. Matthew Elrod-Erickson, Dr. Rebecca L. Seipelt-Thiemann, and Dr. Paul C.Kline. They gave me their advice as my project evolved and aided in shaping the final product both inside and outside of class, and I am very grateful for their assistance.

I would like to further acknowledge my colleagues, Hyo "Erin" Park, and Corbett C. Ouellette. Erin was the hinge of many of my experiments, learning ELISAs, Westerns and Flow Cytometry from me and then becoming expert I technical skill herself. She has put in innumerable hours of work on this project alongside me, and I am deeply grateful to her being a reliable colleague and friend. As Corbett often likes to say he chose our lab after speaking with me about my project.

Ironically, at the time he could explain the project to the layperson far better than I could. He put in many hours of work side by side with me and I appreciate his continuous willingness to help and his ambition to achieve whatever goal was set in front of him.

Finally, I want to thank the other Molecular Biosciences Ph.D. students and the Biology Department staff both faculty and office. Though I could spend many more pages listing the exploits we all had together, I feel than many of you are the reason I am who I am today, and I want to thank you for your support, encouragement, and help over the years.

## ABSTRACT

*Gardnerella vaginalis* is a Gram-positive bacterium associated with BV (BV), pelvic inflammatory disease, and preterm birth. BV is the most prevalent vaginal infection in women, characterized by the absence of normal *Lactobacilli* and overgrowth of *G. vaginalis* and other bacterial species. This study tested the hypothesis that *G. vaginalis* induces an inflammatory response in the human cell line, THP-1. The objectives of the study were to 1) determine whether different strains of *G. vaginalis* cause proinflammatory cytokinesproduction in THP-1 cells, 2) characterize intracellular pathways by which these cytokines are produced, and 3) determine molecular mechanisms involved in death of cells treated with strains of *G. vaginalis.*

In these studies, *G. vaginalis* strain 14018 induced statisticaliy significant increases in the inflammasome-dependent cytokines IL-1β, IL-18, as well as TNF-α in THP-1 monocytes. This same strain of *G. vaginalis* also caused statistically significant cell death in THP-1 monocytes and cleavage of caspase-1 by 24 h following treatment. Knockdown of the inflammasome component, NLRP3, in THP-1 cells reduced secretion of IL-1β. Additionally, THP-1 cells stably expressing ASC with a fluorescent tag exhibited colocalization of NLRP3 with ASC in *G. vaginalis*-treated THP-1 cells. These studies confirmed the role of the NLRP3 inflammasome in *G. vaginalis* inflammation.

In a strain-specific study, a statistically significant increase in THP-1 monocyte differentiation and IL-1β secretion were detected in response to *G.*

*vaginalis* strains 14018 and 49145 but not strain 14019. Cytokine and inflammasome responses were similar for strains 14018 and 49145, but strain 14019 did not induce an inflammatory response in THP-1 cells.

The strain-specific ability of *G. vaginalis* to induce inflammation could contribute to the variability observed clinically between women colonized with *G. vaginalis*. The deleterious effects of *G. vaginalis* observed in THP-1 monocytes were not observed for the human trophoblast cell line HTR8 when treated with any of the *G. vaginalis* strains.

The results of these studies increase the understanding of how *G. vaginalis* activates the innate immune system and suggests that a strain-dependent activation of inflammation may be involved in BV and preterm birth.

## TABLE OF CONTENTS

## LIST OF FIGURES

# INTRODUCTION

## *Gardnerella vaginalis*: BIOLOGY OF THE BACTERIA

*Gardnerella vaginalis*, is a small, Gram-variable bacterium discovered by Leopold in 1953 (Leopold, 1953), then characterized by Gardner and Dukes in 1955 (Gardner and Dukes, 1955) as the organism responsible for nonspecific BV. It was initially placed in the *Haemophilus* genus, based on initial Gram-negative staining and fastidious growth. The first electron micrographs by Criswell et al. revealed that *G. vaginalis* contains what appeared as a Gram-negative cell wall structure (Criswell et al., 1972). Initially, *G. vaginalis* seemed to possess gram-negative structures and was thought to contain lipopolysaccharide (LPS), but followup studies showed a lack of LPS, although an endotoxin-like molecule was present (Sadhu et al., 1989). Still later, a lack of requirement for X and V factor, in addition to emerging evidence that gram-positive structures were also present, caused *G. vaginalis* to be moved to the *Corynebacterium* genus (Catlin, 1992). Following genetic analysis and DNA-DNA hybridization, it was discovered that *G. vaginalis* shared little sequence homology or biochemical characteristics with either the *Haemophilus* or *Corynebacterium* genus (Piot et al., 1980), prompting the formation of a separate genus named for Gardner (Greenwood, 1983).

Initial claims suggested that up to 90% of women with bacterial vaginosis (BV) were colonized with *G. vaginalis,* but these investigators failed to analyze

healthy women for colonization (Gardner and Dukes, 1955). Inoculation of *G. vaginalis* into three healthy women caused only one of them to develop BV, although the other two were colonized. However, pregnant women developed BV nearly 25% of the time when previously colonized with *G. vaginalis* (Koumans et al., 2007). Inoculation of vaginal secretions from women with BV into healthy women caused the development of BV in more than 70% of patients. Upon improvement of culture media for *G. vaginalis*, it was demonstrated that 50% to nearly 100% of healthy women were colonized with *G. vaginalis*, implicating it as an opportunistic commensal rather than an overt pathogen (Zozaya-Hinchliffe et al., 2010).

BV is a condition notable for a purulent discharge, a foul-smelling fish-like odor, the presence of clue cells (viable epithelial cells covered in bacteria) from PaPanicolaou tests (pap smear), and a replacement of the normally dominant *Lactobacillus* species with *G. vaginalis, Mycoplasma hominis, Atopobium vaginae* and *Prevotella, Peptostreptococcus, Bacterioides,* and *Morbilinus* spp. (Turovskiy et al., 2011). These changes in species may be associated with a pH change within the vaginal tract, that occur during menstruation, tampon use, and pregnancy; which may also explain why the prevalence of BV in women is highly variable, depending on the population (Moreira Mascarenhas et al., 2012).

*G. vaginalis* is present in up to 90% of cases of BV, suggesting its greater pathogenicity compared to the other colonizing species, which is the primary rationale for using *G. vaginalis* in this study. Within the course of BV, mucosal

IgA against *G. vaginalis* is observed and is proposed to be added to the BV clinical criteria (Cauci et al., 2002). In addition, *G. vaginalis* has caused bacteremia (Lagace-Wiens et al., 2008) and is associated with preterm birth (Onderdonk et al., 2003), pelvic inflammatory disease (Sharma et al., 2014), endometriosis (Swidsinski et al., 2013), urinary tract infections (Pedraza-Avilés et al., 2001), and an increased risk of sexually transmitted infections (STIs), particularly HIV (Olinger et al., 1999). Risk factors for acquiring BV include vaginal douching (Luong et al., 2010), African ethnicity (Peipert et al., 2008), cigarette smoking, low socioeconomic status, and multiple sexual partners (Nelson et al., 1998). This may be partially explained by growth of *G. vaginalis* within the male urethra (Virecoulon et al., 2005), but also by frequency of intercourse, oral sex, and non-penetrative digito-genital contact, suggesting that *G. vaginalis* may be a sexually enhanced disease (Swidsinski et al., 2010).

*Lactobacillus* bacteria of the vaginal tract are known to inhibit the overgrowth of *G. vaginalis* most likely by production of hydrogen peroxide and acids contributing to a lower pH (approximately pH 4) inhibitory to the growth of *G. vaginalis* (Skarin and Sylwan, 1986). *Lactobacillus* bacteria are also capable of producing bactericidal peptides that specifically bind to cytoplasmic membranes of *G. vaginalis* and dissipating the proton motive force (Turovskiy et al., 2009). *G. vaginalis* is also capable of forming biofilms containing multiple species of organisms and is generally the most predominant organism within these biofilms. These biofilms contain approximately $1x10^9$ organisms of varying

composition, but of those bacteria, *G. vaginalis* is the only organismcapable of biofilm formation (Swidsinski et al., 2008). Additionally, the bacteria is capable of epithelial cell cytotoxicity, which is not observed with other bacteria found in BV biofilms. This biofilm formation also provides *G. vaginalis* resistance to orally-administered metronidazole, the drug of choice for BV therapy (Swidsinski et al., 2008).

Isolation of *G. vaginalis* requires selective and differential media because of the polymicrobial environment of the vagina. Many varieties of growth media have been developed incorporating antibiotics such as gentamyicin, amphotericin B, and nalidixic acid to which *G. vaginalis* is resistant (Catlin, 1992). The main component of most media prepared specifically for *G. vaginalis* culture is human blood, on which it will cause β-hemolysis. The hemolytic activity is caused by a cytolytic toxin, secreted by *G. vaginalis* and first described by Rottini et al. (1990) as an ampipathic molecule with a molecular mass of 61 or 63 kD. *Gelber* et. al (2008) characterized the toxin, termed vaginolysin, as being a member of the cholesterol-dependent cytolysins (CDC), a group of pore-forming toxins known to oligimerize with cholesterol within eukaryotic membranes. Vaginolysin is specific for human cells, particularly human erythrocytes for which it has at least a 1000-fold increase in hemolytic activity. This specificity is the result of binding to human CD59 (hCD59), a finding also reported for a closely homologous CDC, intermedilysin of *Streptococcus intermedius* (Johnson et al., 2013). CDC cytolysins can interact with intracellular effectors, and some of these toxins can

act as inflammasome modulators, supporting the need for better understanding of *G. vaginalis* and its toxin and their interaction with the innate immune system.

## Inflammasomes: MEDIATORS OF INFLAMMATION

Inflammasomes are multiprotein complexes that oligimerize to activate the enzyme caspase-1, which then leads to the processing and secretion of the proinflammatory cytokines interleukin-1 (IL-1) and interleukin-18 (IL-18). Four distinct inflammasomes have been identified, each of which is triggered by a unique subset of ligands with minimal overlap, suggesting a stringent activation mechanisms. The key protein of these inflammasomes is an intracellular pattern recognition receptor (PRR). Three of the inflammasomes (Fig. 1) contain PRRs belong to the NOD (nucleotide binding and oligimerization domain) and LRR (leucine-rich repeat)-containing receptor (NLRfamily. The fourth inflammasome contains AIM2 (Absent In Melanoma 2), which uses a binding mechanism involving apyrin domain functionally similar to that of the NLR family, although AIM2 is a member of the hematopoietic interferon-inducible nuclear protein (HIN) family (Hornung et al., 2009).

Activation of inflammasomes occurs in five steps. 1) Following binding to the activating ligand, 2) the PRR binds to the linker molecule ASC (Apoptosis-associated Speck-like protein containing a CARD domain) through the pyrin domain, and 3) this PRR/ASC complex then binds to procaspase-1 through the

interactions between the CARD domains. That event results in 4) the proteolytic cleavage of the caspase-1 pro-domain resulting in a catalytically active heterotetramer of two p20 and two p10 subunits. This enzyme 5) cleaves pro-IL-1β and pro-IL-18 to mature IL-1β and IL-18, resulting in an inflammatory response. Very high activation of caspase-1 causes it to cleave and activate the initiator caspase-7, which in turn cleaves and activates the executioner caspase-3 and leads to an apoptosis-like event, termed pyroptosis (Brodsky and Medzhitov, 2011). Due to this complex mechanism observed in the recruitment of the inflammasome, different pathogen associated molecular patterns (PAMPs), such as bacterial lipopolysaccharide (LPS), and damage associated molecular patterns (DAMPs), such as ATP release, will result in different inflammatory signals (Gross et al., 2011). Exposure to these danger signals (e.g. LPS±ATP) can serve as positive controls for inflammasome activation, since these compounds will individually cause low level recruitment and together result in high level recruitment of some inflammasomes (Miao et al., 2011).

The NLR family of inflammasome proteins has three characteristic domains, an N-terminal effector domain for signal transduction, a nucleotide binding and oligimerization domain (NOD, Nacht), and a C-terminal leucine-rich repeat (LRR) domain (Schroder and Tschopp, 2010). The NLR family of proteins (Fig. 1), is known for its ancestral role in innate immunity through the recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). The N-terminal domains of the protein family

contain pyrin or CARD (caspase recruitment domain) domains (Fig. 1). The pyrin domain is predicted to contain secondary structure very similar to death domains (DD), death-effector domains (DED), and caspase recruitment domains (Fairbrother et al., 2001). The pyrin domain combined with its homology to the known caspase 3 regulator Apaf-1 seems to suggest that this domain may play a key role in regulating cysteinyl aspartate protease (caspase) function (Hiller et al., 2003). The pyrin domain is associated with inflammation and demostrates a connection to the inflammatory caspase family, i.e. caspases 1, 4, 5, and 12 (Martinon and Tschopp, 2007).

Inflammasomes are activated by multiple signals, including bacterial and viral structural components, toxins, and nucleic acids. Following binding of the NLR or AIM2 component to its ligand, the protein then oligmerizes with the adapter protein ASC. ASC forms an adaptor between the CARD domain on caspase-1 and the pyrin domain on NLRP3, NLRC4, or AIM2 (Hornung et al., 2009;Mariathasan et al., 2004). In some cases, it seems as though ASC can form a very large homo-multimeric complex of its own leading to cell death. Normally, oligimerization is inhibited by a high intracellular potassium concentration, which explains why P2X7 potassium channel activation or membrane penetrating toxins cause activity (Franchi et al., 2007). ASC is able to recruit NF-κB and induce the expression of IL-8 (Hasegawa et al., 2005), likely through a second messenger. Because of its domains, it is possible that ASC represents a sort of central regulator, capable of causing or preventing

inflammatory cytokines based on expression levels or inhibition by other CARD containing proteins like pyrin-only protein-2 (POP2) or pyrin itself (Bedoya et al., 2007).

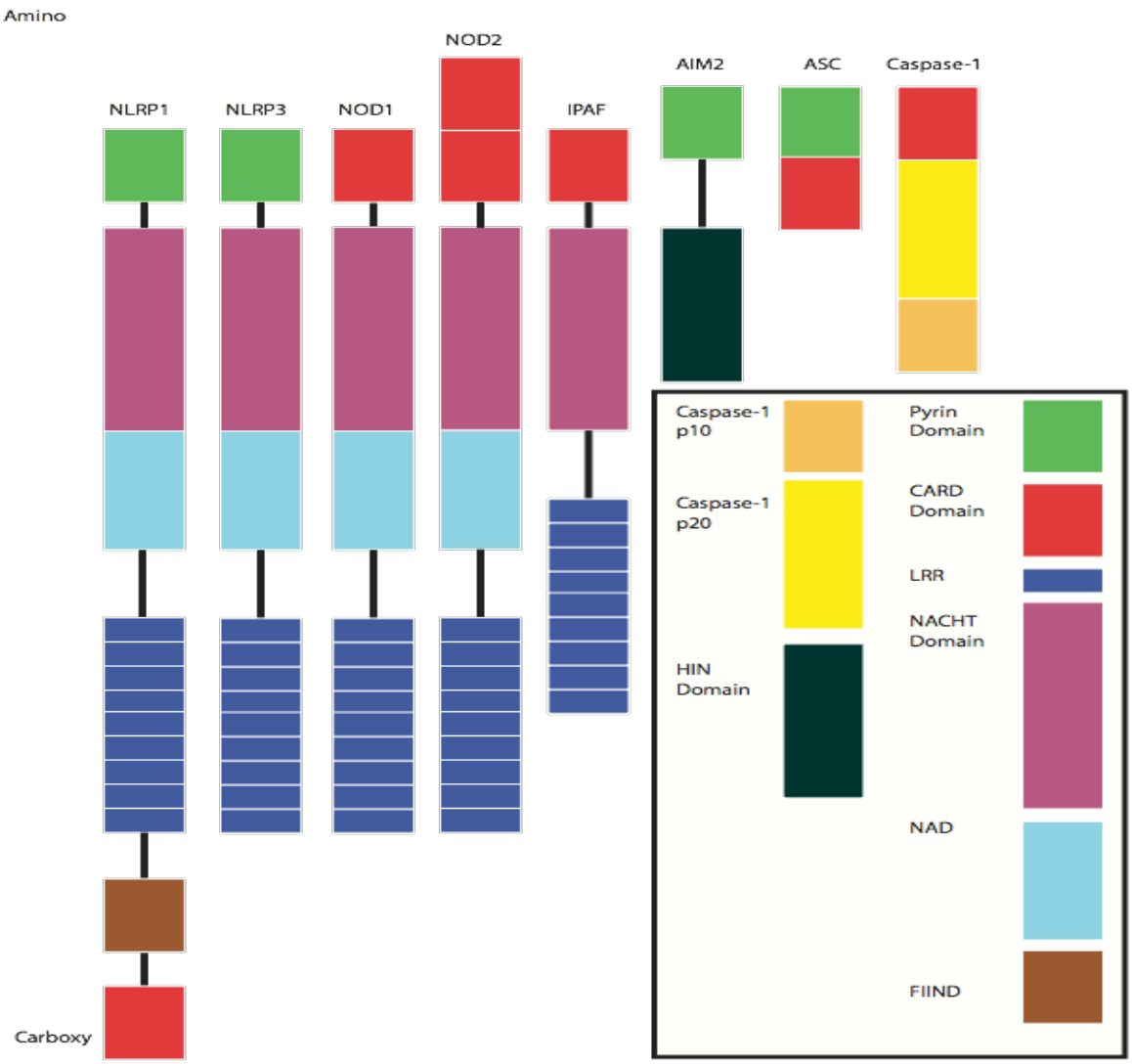



**Figure 1.1: Comparisons of the members of the NOD-like family of proteins** The NLR Family (right) contain a N-terminus pyrin or CARD domain, resulting in differential combination and variation in inflammasome recruitment. NLRP1 and NLRP3 contain an N-terminal pyrin domain, leucine-rich repeats (LRR), NACHT domain, NACHT Associated Domain (NAD), with NLRP1 containing a CARD and FIIND domain (allowing it to have a more diverse

association).

The interaction between the CARD domain of ASC and the CARD domain of inflammatory caspases results in the formation of active caspases. Caspases have been well characterized within the context of apoptosis. Thirteen caspases are known in humans, and these can be divided into initiator caspases, executioner caspases, and inflammatory caspases. Initiator caspases rely on adjacent proteins, which contain a receptor domain or interact with a receptor secondarily, as is the case with Fas and TRAIL (Lamkanfi and Kanneganti, 2010). Some initiator caspases possess a death effector domain (DED) or a caspase activation and recruitment domain (CARD), allowing them to interact directly with their receptors (Alcivar et al., 2003). Most caspases in general contain a pro-domain, which will be cleaved in order to confer activity. In the case of executioner caspases, this occurs as the result of activation of initiator caspases, e.g. Apaf-1 causes the cleavage of caspase-9 (an initiator caspase) which leads to the formation of a large, multimeric complex formed between Apaf-1 (an adaptor molecule with a CARD domain), caspase-9, and cytochrome c. Together they cause the cleavage of caspase-3 (an executioner caspase), which subsequently acts on many downstream effectors such as iCAD, PARP, and many other substrates initiating apoptosis (Zou et al., 2003). The Apaf-1 interaction has been shown to be very similar to the mechanism of NLR-mediated inflammasome activation but utilizes inflammatory caspase activation instead of executioner caspase activation (Chu et al., 2001).

Of the inflammatory caspases (caspase-1, caspase 4, caspase-5, and caspase-12), caspase-1 (IL-1β Converting Enzyme) is the best understood in terms of mechanism. All of the inflammatory caspases are synthesized as inactive zymogens and gain catalytic activity upon cleavage. Caspase-1 and caspase-5 are expressed at low levels in most tissues but are constitutively expressed in higher levels in cells of myeloid lineage especially monocytes, macrophages, dendritic cells and to a lesser extent in neutrophils. Caspase-1 recognizes IL-1β, IL-18, and IL-33 as substrates, cleaving their pro-domains and conferring biological activity. Each of these cytokines has a wide reaching inflammatory activity. IL-1β is pyrogenic, causes expression of cyclooxygenase-2 (COX-2) in neurons, and nitric oxide production. IL-18 causes activation of natural killer cells and production of interferon gamma. Sustained activation of caspase-1 is the cause of pyroptosis, a form of cell death associated with a large release of inflammatory cytokines (Fantuzzi and Dinarello, 1999).

The inflammasome NLRP3 (Nalp3, cryopyrin, Caterpillar1.1, CIAS1) belongs to the NLR inflammasome family and is one of the best-known inflammasome mediators. It is recruited by the widest array of substrates and well characterized in terms of its role in human disease. NLRP3 is also known as cryopyrin because it is associated with auto-inflammatory periodic syndromes, a series of three genetically inherited conditions characterized by increased or unregulated levels of IL-1β with very similar but distinct phenotypes (Kambe et al., 2010). Familial cold auto-inflammatory syndrome (FCAS), Muckle-Wells

syndrome, and chronic infantile neurological, cutaneous and articular (CINCA) syndrome are all associated with heterozygous cryopyrin mutations, usually within the NACHT (NOD) domain. Therapy consists primarily of administering IL-1β inhibitors such as anakinra, indicating that NLRP3 has a key regulatory role in managing IL-1β (Dowds et al., 2004). NLRP3 recognizes a very diverse range of inflammatory mediators, including monosodium urate (Martinon et al., 2006), asbestos, silica (Dostert et al., 2008), and low intracellular potassium (Pétrilli et al., 2007), along with a large array of microbial triggers, which show specific activation profiles (Martinon and Tschopp, 2007). A growing number of microbial sources and their toxins seem to either activate or regulate the NLRP3 inflammasome including *Mycobacterium tuberculosis* protein ESAT-6 (Mishra et al., 2010)*, Candida albicans* (Hise et al., 2009)*, Staphyloccocus aureus* alpha hemolysin (Craven et al., 2009), *Pseudomonas aeruginosa* pilin (Arlehamn and Evans, 2011)*, Streptococcus pyogenes* (Harder et al., 2009)*,* HIV-1 (Guo et al., 2014)*,* and adenovirus type 5 (Barlan et al., 2011), among others. Many of the activating substrates for the other three inflammasomes are also known. The NLRP1 inflammasome is recruited by *Bacillus anthracis* lethal toxin and muramyl dipeptide of bacteria (Averette et al., 2009). The AIM2 inflammasome is stimulated by double stranded DNA in the cytosol and is responsible for the development of inflammatory response to viruses, bacterial DNA, and in some cases one's own DNA (Choubey, 2012). NLRC4 (IPAF) will recruit an inflammasome to a similar diversity of substrates, with its most well-known

activator being intracellular flagellin, and is capable of being co-recruited or being recruited to a poly-NLR inflammasome with NLRP3 (Lage et al., 2014; Man et al., 2014).

As their name suggests, inflammasomes are critical components of inflammation and the regulation of inflammatory secretions and processes. Recently, they have been the target of toxicological, bacteriological, viral, and biochemical analyses to establish which effectors do and do not demonstrate activity. However, many aspects of their activation, especially in the signal cascade which recruits the inflammasome, still remain elusive. In the present study, the inflammasome is used to determine the possible mechanism by which *G. vaginalis* can cause cytokine secretion and cell death.

**Specific Aims**

Due to its prevalence, *G. vaginalis* obviously has an important role in the immunopathology of the genital mucosa, but much of that role remains unclear. Women with BV have been known to secrete IL-1β, IL-18, and TNF-α (Zaga-Clavellina et al., 2012). By focusing on an *in vitro* system of *G. vaginalis* in association with macrophages, cells that would most likely cause inflammasome recruitment and cytokine release in the host, it may be possible to demonstrate at a molecular level that *G. vaginalis* is capable of recruiting the inflammasome and causing inflammation. A basic understanding of the capacity of *G. vaginalis* to

cause inflammation may further explain the roles for this organism in the still poorly understood etiology of BV and preterm birth.

Hypothesis: *G. vaginalis* activates the NLRP3 inflammasome in THP-1 macrophage-like cells.

This hypothesis was tested through the following specific aims:

1) Identify a cytokine profile in THP-1 cells exposed to *G. vaginalis* strains that is indicative of inflammasome activation.

THP-1 cells were exposed to LPS (PAMP), ATP (DAMP), LPS and ATP, and *G. vaginalis* (MOI 5) and then assayed by ELISA for the production of inflammasome-dependent cytokines. The inflammatory profile of infected and uninfected cells was characterized to determine the cytokines produced in response to the bacterium. These investigations included comparisons between American Type Culture Collection (ATCC) strains 14018, 14019, and 49145 of *G, vaginalis.*

2) Identify the inflammasome components recruited in response to treatment with *G. vaginalis* strains to determine the involvement of the NLRP3 in inflammasome activation

Fluorescent antibodies directed to inflammasome components were used to detect co-localization between NLRP3, or another imflammasome component NLRC4, and a fluorophore-labeled ASC. Additionally, the knockdown of NLRP3 was used to confirm that NLRP3 was both necessary and sufficient for caspase-1 activation and the release of the inflammasome product IL-1β. Co-localization studies and activation of inflammasome components was also compared for strains 14018, 14019, and 49145 of *G. vaginalis.*

Identify the mechanism of cell death in response to *G. vaginalis* treatment. Preliminary results suggested that *G. vaginalis* treatment of THP-1 cells results in significant cell death, the capacity of *G. vaginalis* to induce pyroptosis was measured by annexin-V assay, caspase-8 activation, and caspase-1 activation by both Western blot and FLICA assays. Treatment of cells with an inhibitor of caspase-1 was also used to assess the decreases in the secretion of inflammasome-dependent cytokines. Measurements of cell death and pyroptosis were compared for both THP-1 and trophoblast cell lines using strains 14018, 14019, and 49145 of *G, vaginalis.*

# CHAPTER I

## *Gardnerella vaginalis* TRIGGERS NLRP3- INFLAMMASOME RECRUITMENT IN THP-1 MONOCYTES

Vick, E.J., Park, H.S., Huff, K.F., Brooks, K.M., Farone, A.L., Farone, M.B., *Gardnerella vaginalis* triggers NLRP3 inflammasome recruitment in THP-1 monocytes. J. Reprod. Immuno Epublished ahead of print. doi:10.1016/j.jri.2014.08.005

### 1. INTRODUCTION

Bacterial vaginosis (BV) is a common vaginal disorder in women of reproductive age and is characterized by an absence of protective *Lactobacillus* spp. and overgrowth of anaerobic and facultative bacteria including *G. vaginalis.* Although the bacterium is observed in asymptomatic women, it occurs in up to 96% of BV cases. *G. vaginalis* has also consistently been observed in conjunction with gynecological and obstetric disease states including pelvic inflammatory disease, endometritis, intra-amniotic infection, miscarriage, premature labor, preterm delivery, fetal brain injury, and acquisition of HIV and other sexually-transmitted infections. Although BV is typically distinguished by a lack of inflammation, TNF-α secretion and upregulation of mRNA for toll-like receptors 2 and 4 were found in women who tested positive for BV. Additionally, women with BV exhibit a 13-fold increase in IL-1β levels, and *G. vaginalis*

induces significant levels of TNF-α in THP-1 monocytes.

During bacterial infections, secretion of IL-1β and another proinflammatory cytokine, IL-18, by monocytes and macrophages is regulated by two distinct signals. First, microbial products and endogenous danger signals from infected or damaged cells are recognized by cytoplasmic pattern recognition receptors (PRR), such as Nod-like receptors. The Nod-like receptor, NLRP3, responds to a variety of stimuli, including bacterial cell wall components, LPS and lipoteichoic acid, and endogenous molecules such as ATP, urate crystals and silica. After binding intracellular ligands, Nod-like receptors associate with the cytoplasmic ASC protein to form the multiprotein complex known as the inflammasome. The inflammasome binds and cleaves caspase-1 to an active form, which subsequently cleaves pro-cytokines IL-1β and IL-18 to their mature forms leading to secretion. Caspase-1 cleavage can occur with inflammasomes containing other members of the Nod-like family of receptors, such as NLRC4, NLRP1, or AIM2, signaled by specific molecules including *Salmonella enterica* flagellin, anthrax lethal toxin, and dsRNA, respectively (Schroder and Tschopp, 2010; Strowig et al., 2012).

Monocytes and macrophages are normally found in the lamina propria of vaginal tissue and not only secrete proinflammatory cytokines and produce several types of PRRs, including Toll-like and Nod-like receptors, but are also capable of assembling different inflammasome complexes in response to a variety of microbial stressors (Schroder and Tschopp, 2010). In this study, we

sought to further elucidate the interaction of *G. vaginalis* with the innate immune system through analysis of THP-1 monocyte cell death, cytokine secretion, and inflammasome formation in response to treatment with *G. vaginalis*.

## 2. MATERIALS AND METHODS

**2.1 Bacteria and co-culture.** *Gardnerella vaginalis* ATCC strain 14018 was cultured on brain heart infusion (BHI) agar (Becton Dickinson) for 48 h, then inoculated into BHI broth and grown until mid-log phase. Bacterial cells were then washed in PBS and resuspended in RPMI 1640 medium (Sigma-Aldrich) before addition to host cells at a multiplicity of infection (MOI) of 5-10.

**2.2 Monocyte culture.** THP-1 monocyte cells (ATCC TIB-202) were cultured in RPMI 1640 supplemented with 10% FBS (Atlanta Biologicals), 50 μM 2-mercaptoethanol, 100 IU penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich) and incubated at 37°C in 5% $CO_2$. ASC-YFP (Yellow Fluorescent Protein) expressing THP-1 cells were the generous gift of Mikhail Gavrillin of the Ohio State University (Columbus, OH, USA). These cells contain a pLenti6/V5 TOPO vector with human ASC fused to the C terminus of yellow fluorescent protein (YFP) resulting in stable expression of ASC (Gavrilin et al., 2012). The shNLRP3 and shCtrl RNAs were a generous gift of the Ting Lab at the University of North Carolina, Chapel Hill and were used to prepare retroviral vectors and THP-1 cells expressing the shRNAs (Duncan et al., 2009; Willingham et al., 2009).

Knockdowns were verified by Western blot with rabbit anti-NLRP3 antibody (Santa Cruz Biotechnology).

**2.3 Measurement of cytokines IL-1β, TNF-α, and IL-18.** THP-1 cells were plated at $5.0 \times 10^5$ cells/ml in six-well tissue culture plates and differentiated with 100 nM phorbol-12-myristate-13-acetate (PMA)(Sigma-Aldrich) before treatment with either fresh pre-warmed medium, medium containing 1μg/ml *Salmonella enterica* LPS (Sigma-Aldrich) and/or 5 mM ATP (Fisher Scientific) (Harder et al., 2009; Costa et al., 2012), 100 μM YVAD (Ac-YVAD-cmk, Sigma-Aldrich), medium containing *G. vaginalis* at an MOI of 5-10 (Costa et al., 2012; Gavrilin et al., 2012; Li et al., 2013;), or medium containing both YVAD and *G. vaginalis.* Cell culture supernatants were collected at 0, 12, and 24 h after treatment and spun at 12,000 × *g* for 5 min at 4°C. Supernatants were collected and cytokines quantified using ELISA kits for IL-1β (Life Technologies), TNF-α (Life Technologies), and IL-18 (MBL International) according to the manufacturer's instructions. ELISA data were analyzed using the SpectraMax M5 microplate reader and SoftMax Pro software (Molecular Devices) and tested for statistical significance by one-way ANOVA using Dunnett's test for multiple comparisons or a two-tailed student *t*-test for individual sample comparison in Prism 6.0 (GraphPad Software).

**2.4 Annexin V staining and flow cytometric analysis.** THP-1 cells were grown to a density of $5.0 \times 10^5$ cells/ml and treated as above. Staurosporine (1$\mu$M; Sigma-Aldrich) was added as a positive apoptotic control. Cells were

stained with Annexin V Alexa Fluor 680 conjugate and counterstained with Sytox Green dead cell stain (Life Technologies) according to the manufacturer's instructions. Briefly, at timepoints of 0, 12, and 24 h, cells were collected, spun at 500 × *g* for 5 min at 4°C, washed in cold PBS, and then resuspended in Annexin V binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4) containing 5µl/ml Annexin V AlexaFluor 680 conjugate and 30 nM Sytox Green. Acquisition was performed on the Millipore Guava 8HT system using Incyte (EMD Millipore) and analyzed using Flowjo software (Tree Star). Statistical analyses comparing cell viability was performed using multiple student's two-tailed *t*-test in Prism 6.0.

**2.5 Immunofluorescent microscopy.** THP-1 cells were grown on sterile coverslips at a density of $5.0 \times 10^5$ cells/ml and treated with either *G. vaginalis* at an MOI of 5-10, LPS/ATP as above, or with medium alone. After 12 h of treatment, all media were removed, and cells were washed with cold PBS and fixed using 3.8% formaldehyde in PBS for 20 min and with PBS containing 5% BSA followed by permeabilization with 0.1% saponin in PBS/BSA for 5 min. Cells were washed twice with PBS/BSA, and then blocked with 5% goat serum (Atlanta Biologicals) in PBS/BSA for 20 min. Primary rabbit anti-NLRP3 antibodies (Santa Cruz Biotechnology) were added in PBS/BSA/5% goat serum (antibody buffer) to the fixed cells followed by incubation at room temperature for 30 min. Coverslips were removed from plates, washed, incubated with goat anti-rabbit IgG AlexaFlour 594 conjugate in antibody buffer in the dark, followed by washing and

post-fixation with 3.8% formaldehyde for 10 min. Prolong Gold antifade-reagent with DAPI (Life Technologies) was added and coverslips were allowed to cure overnight at 4°C and then imaged using a Nikon F2000 laser scanning confocal microscope and EZ-C1 acquisition software.

**2.6 SDS-PAGE and Western blot analysis.** THP-1 cells were plated at 5.0 × $10^5$ cells/ml, differentiated as above, and treated with *G. vaginalis* at an MOI of 5-10, LPS/ATP, or medium alone, and incubated as before. After 12 h, cells were lysed with cold RIPA buffer with 1X protease inhibitors (Sigma-Aldrich). Lysates were centrifuged at 12,000 × *g* for 5 min at 4°C, supernatants were collected, and protein concentration was quantified by Bradford assay (ThermoFisher Scientific). To visualize excreted caspase-1, THP-1 cells were plated at 5.0 × $10^5$ cells/ml and equivalent volumes of cell medium were precipitated with 10% TCA at 24 h, incubated on ice for 15 min, and pelleted at 18,620 *x g* for 15 min at 4°C. Pellets were washed with cold acetone, resuspended in Laemmli buffer, and boiled for 5 min. Proteins were resolved on 4-12% SDS-PAGE gels (Life Technologies) and transferred to a nitrocellulose membrane using the iBlot dry blotting system (Life Technologies) or to PVDF using the XCell II system (Life Technologies). Membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) then probed with the following primary antibodies: mouse anti-caspase 8 p18, mouse anti-β-actin (from Santa Cruz Biotechnology), or capsase-1 p20 antibody (Adipogen) in 2.5% nonfat milk TBS-T. After washing, blots were probed with donkey anti-rabbit or anti-mouse

horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and visualized with ECL according to manufacturer's instructions (Pierce Biotechnology). Imaging was performed on a Bio-Rad EZ imager and analyzed with Bio-Rad Image Lab software.

**2.7 Fluorescent-labeled inhibitors of caspase assay (FLICA)** THP-1 cells were plated at $5.0 \times 10^5$ cells/ml, differentiated as before, and treated with *G. vaginalis* at an MOI of 5-10, LPS/ATP, YVAD, YVAD and *G. vaginalis*, or medium alone, and incubated as above. The caspase-1 FLICA 660 kit (Immunochemistry Technologies) was used according to manufacturer's instructions. Cells were removed from plates by cold 5 mM EDTA in PBS, and then quantified by flow cytometry as above.

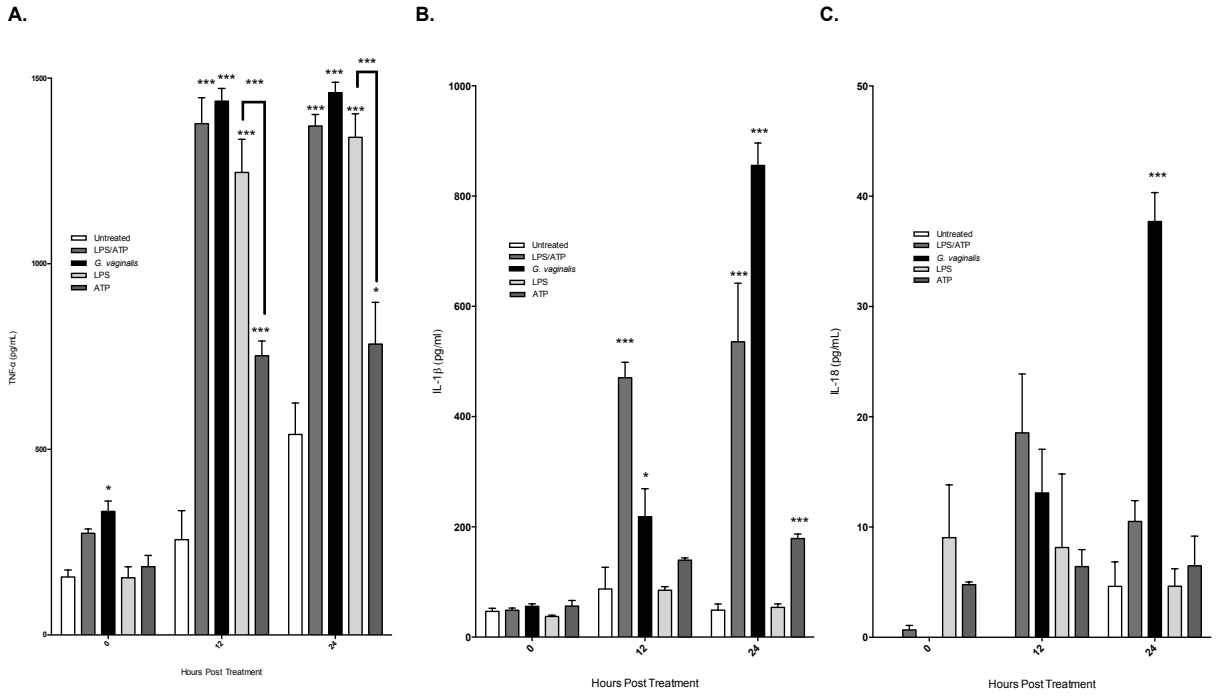



**Figure 2.1 Cytokine secretion follows *G. vaginalis* treatment of THP-1 cells.** 5 x $10^5$ cells/ml of PMA-differentiated THP-1 cells were incubated with *G. vaginalis* at an MOI of 5-10, 1 µg/ml LPS, 5 mM ATP, LPS/ATP in combination, or fresh medium (untreated). Supernatants were collected from each control or treatment group at 0, 12, and 24 h and quantified for cytokine levels by ELISA. (A) By 12 h, treatment with *G. vaginalis*, LPS, ATP, or LPS/ATP resulted in significant increases in the TNF-α levels compared to untreated cells. The TNF-α levels with *G. vaginalis*, LPS, and LPS/ATP treatments were significantly greater than ATP treatment alone. These cytokine levels persisted through 24 h. (B) IL-1β production increased significantly in both the LPS/ATP control and *G. vaginalis* treated cells by 12 h. IL-1β continued to increase significantly through 24 h post treatment in these cells along with ATP-treated cells. (C) IL-18 cytokine

release in LPS/ATP and *G. vaginalis* treated cells was significantly increased by 12 h, and by 24 h only *G. vaginalis* treated cells produced significant levels of IL-18 compared to both medium alone and LPS/ATP controls. All results are the means ± S.E. ($n \geq 3$) of biological replicates and are representative of at least two independent experiments. Statistical differences between groups were determined by a two-tailed t test (*$p < 0.05$, **$p < 0.01$, ***$p<0.001$).

## 3. RESULTS

### 3.1 *G. vaginalis* induces proinflammatory cytokines in THP-1 cells

Initially, we asked whether *G. vaginalis* could induce the expression of the inflammatory cytokines TNF-α, IL-1β, and IL-18 in THP-1 cells. LPS and/or ATP treatments were used as controls. Treatments with LPS and ATP were used as positive controls for NLRP3-mediated inflammasome activation. After incubation with either LPS/ATP or *G. vaginalis* (Fig. 1A), TNF-α release peaked at 12 h after treatment, consistent with an immediate inflammatory insult, and remained elevated compared to the untreated control until the 24 h time point. IL-1β peaked after 12 h of LPS/ATP exposure and was significantly increased in *G. vaginalis* treated THP-1 cells at 24 h with no significant difference between *G. vaginalis* and LPS/ATP treated cells ($P$=0.120) (Fig. 1B). IL-1β secretion increased significantly between 12 and 24 h after *G. vaginalis* treatment ($P$=0.006). Although ATP stimulation of LPS-primed cells can result in cytokine production within 30 min, the time course used in this study reflects the 24 h time period necessary for maximal IL-1β response (Grahames et al., 1999; Perregaux et al., 2000; Mehta et al., 2001). Similar IL-1β response times have also been observed for other bacterial treatments of macrophages (Monack et al., 2001; Atianand et al., 2011; Park et al., 2014). By 24 h, IL-18 secretion was also significantly increased in *G. vaginalis* treated cells compared to both untreated and LPS/ATP treated THP-1 cells ($P$=0.013, $P$=0.001 respectively) (Fig. 1C).

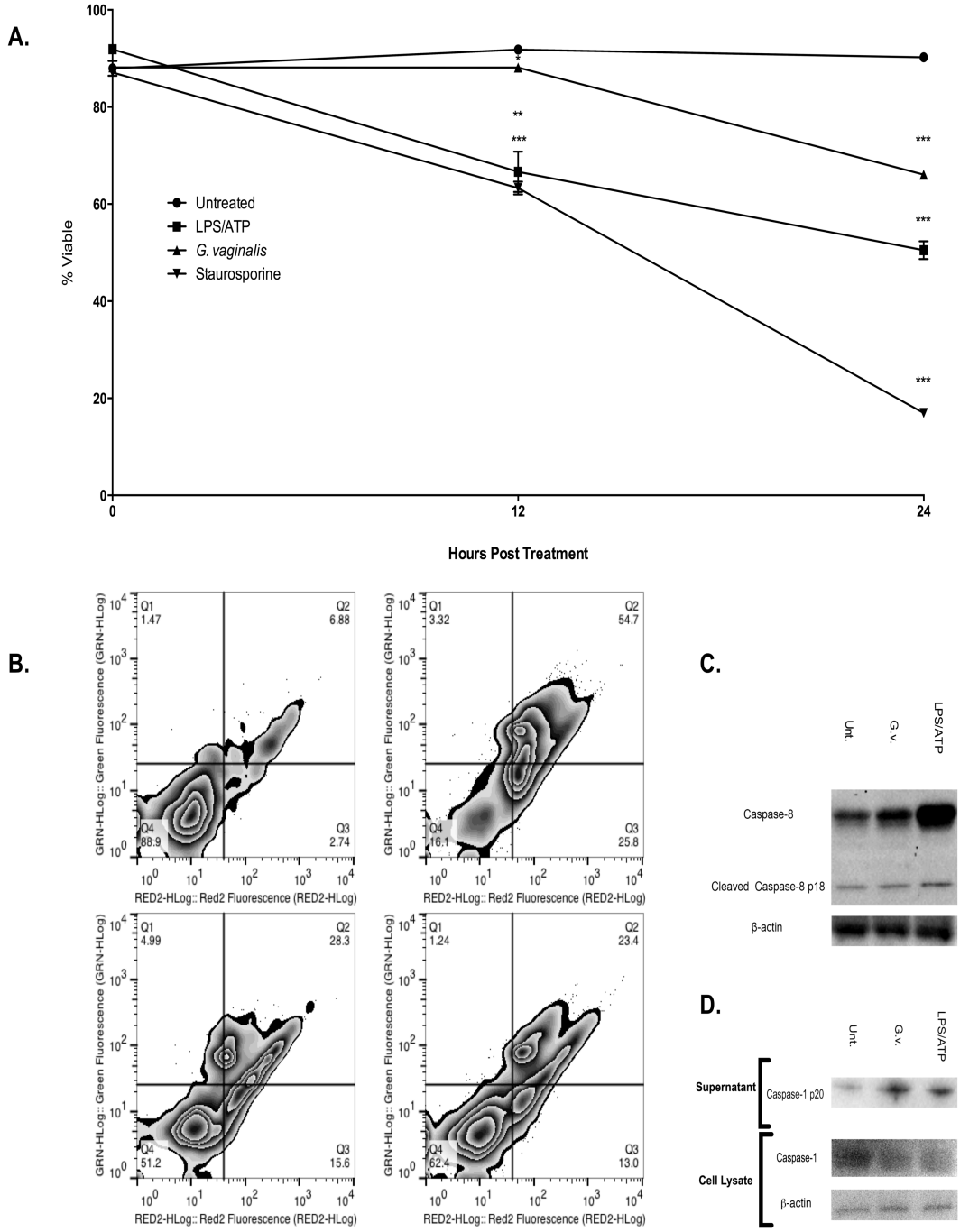



**Figure 2.2 *G. vaginalis* induces THP-1 cell death through pyroptosis.** 5 x $10^5$ THP-1 cells/ml were treated with *G. vaginalis* at an MOI of 5-10, 1 µg/ml

LPS/5 mM ATP, 1 μM staurosporine as an apoptosis control, or medium alone (untreated). Cells were collected at 0, 12, and 24 h and stained with AnnexinV AlexaFluor 680 conjugate and Sytox Green and analyzed by flow cytometry for membrane asymmetry and integrity. (A) LPS/ATP and staurosporine treatment resulted in significant decreases in viability by 12 h post treatment compared with untreated cells. The viability of *G. vaginalis* treated cells was not significantly different from untreated. By 24 h, cell viability significantly decreased to 17.0% with staurosporine treatment, 50.5% with LPS/ATP treatment, and 66% in the *G. vaginalis* treated group. All treatments were significantly different compared to untreated cells, which remained at 88.9% viability (*$p < 0.05$, **$p < 0.01$, ***$p<0.001$). (B) (Clockwise from the top left: Untreated, Staurosporine, *G. vaginalis,* LPS/ATP) Representative flow cytometric analysis and percent of cells positive for Annexin V (bottom right quadrant), Annexin V and Sytox Green (top right quadrant), and Sytox Green (top left quadrant). At 24 h, the majority of cells in all but the untreated group are double-stained indicating that the cells are either late apoptotic or pyroptotic. Results are the mean ± S.E. ($n \geq 3$) of biological replicates and are representative of at least two independent experiments. (C) $5 \times 10^5$ THP-1 cells/ml were treated for 12 h with *G. vaginalis* at an MOI of 5-10, 1 μg/ml LPS/5 mM ATP, or medium alone (untreated). Cells were lysed and supernatants were collected for protein assay, electrophoresis, and Western blotting. Caspase-8 is upregulated in treatment groups but no significant caspase-8 cleavage is observed in *G. vaginalis* treated cells. (D) For

detection of caspase-1 cleavage, 5 x $10^5$ THP-1 cells/ml were treated for 24 h as above. Protein in cell supernatants was precipitated with 10% TCA before protein assay, electrophoresis, and transfer for Western blots. Supernatants possessed increased caspase-1 p20 cleavage product in *G. vaginalis* treated cells, compared to untreated samples. Caspase-1 p20 cleavage was comparable between the LPS/ATP control and *G. vaginalis*, further demonstrating pyroptosis, while cell lysates from those experiments showed native caspase-1 depletion in *G. vaginalis* and LPS/ATP treatments.

### 3.2 *G. vaginalis* induces programmed cell death in THP-1 cells

Inflammasome activation, along with IL-1β and IL-18 release, also triggers a form of cell death known as pyroptosis in monocytes and macrophages (Bergsbaken et al., 2009). We wanted to address whether the proinflammatory cytokine response to *G. vaginalis* resulted in THP-1 cell death. Using Alexa Fluor 680-conjugated Annexin V and Sytox Green staining with flow cytometry, a significant increase in cell death was observed in both staurosporine ($P = 0.002$) and LPS/ATP treated ($P = 0.025$) cells by 12 h, whereas *G. vaginalis* treated cells remained 88% viable (Fig. 2A). At 24 h, viability of *G. vaginalis* treated THP-1 cells decreased significantly compared to untreated control cells to 66% ($P = 0.003$), but the amount of cell death was significantly less than cells treated with LPS/ATP ($P = 0.042$) or the apoptotic inducer staurosporine after 24 h ($P = 0.001$). The delayed decline in cell death of the *G. vaginalis* treated cells suggests that *G. vaginalis* does not cause an immediate cell death, but allows time for cells to generate a significant and ongoing cytokine response.

Using a 4-quadrant gate of the stained cells, the majority of the dead cells treated with staurosporine, LPS/ATP, or *G. vaginalis* were both Annexin V and Sytox Green positive by 24 h (Fig. 2B), indicating phosphatidyl serine exposure characteristic of both apoptosis and pyroptosis, as well as membrane damage associated with both pyroptosis and the dead cells of late apoptosis (Fink and Cookson, 2006). To better characterize the delayed cell death observed in *G. vaginalis*-treated cells, caspase-1 and caspase-8 activation were measured in

treated cells. Caspase-1 cleavage was significantly increased in both LPS/ATP and *G. vaginalis* treated cells (Fig. 2D). Although levels of caspase-8 were upregulated in LPS/ATP treated samples, no significant differences were observed in the amount of cleaved caspase-8 between treated and untreated samples (Fig. 2C). These data suggest that cell death is the result of pyroptosis and not a TNF-α mediated extrinsic pathway of apoptosis.

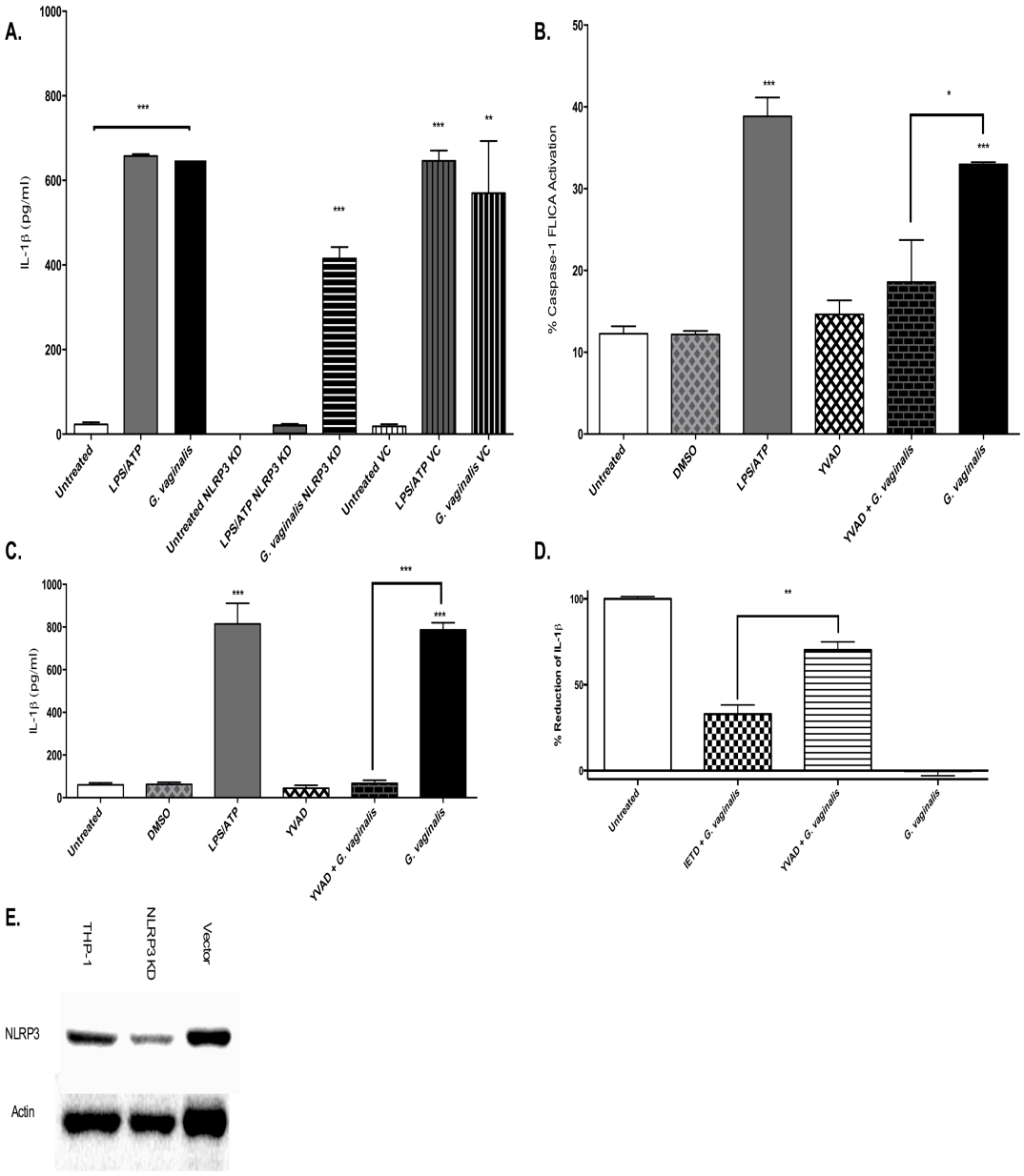



**Figure 2.3 *G. vaginalis* induced IL-1β release requires caspase-1 activation and NLRP3 inflammasome activation.** (A) THP-1 cells, THP-1 cells stably transduced with NLRP3-specific shRNA (shNLRP3 knockdown, KD), or THP-1 cells transduced with the vector control shRNA (shCtrl, VC) were treated with *G. vaginalis* at an MOI of 5-10, 1 μg/ml LPS/5 mM ATP, or medium alone

(untreated). (A) NLRP3 depletion caused no significant reduction in levels of TNF-α. (B) IL-1β release was significantly reduced in knockdown THP-1 cells treated with LPS/ATP or *G. vaginalis* indicating the necessity of NLRP3 for *G. vaginalis* inflammasome recruitment. THP-1 cells were then treated with *G. vaginalis* at an MOI of 5-10, 1 µg/ml LPS/5 mM ATP, 100 µM Ac-YVAD-cmk (YVAD), DMSO, or medium alone (untreated). (C) The addition of the caspase-1 inhibitor Ac-YVAD-cmk, completely abrogated IL-1β secretion in cells treated with *G. vaginalis*. (D) Additionally, caspase-1 specific inhibition was verified by FLICA staining using flow cytometry. (E) NLRP3 knockdown was confirmed by Western blot. Results are the mean ± S.E. ($n \geq 3$) and are representative of at least two independent experiments. Statistical differences between groups were determined by a two-tailed t test (*$p < 0.05$, **$p < 0.01$, ***$p<0.001$).

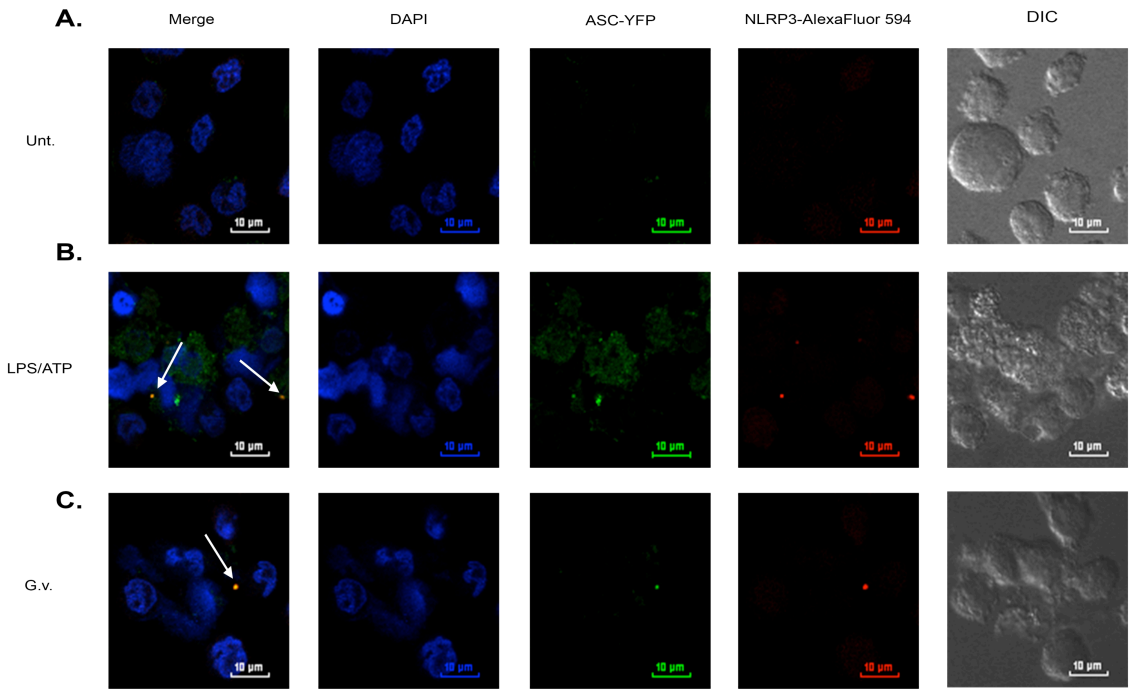



**Figure 2.4 NLRP3 colocalization with YFP-ASC in THP-1 cells reveals the NLRP3 inflammasome.** THP-1 cells stably expressing YFP-ASC were grown on sterile coverslips and treated with *G. vaginalis* at an MOI of 5-10, 1 µg/ml LPS/5 mM ATP, or fresh medium (untreated) for 12 h before fixation and immunofluorescent staining with anti-NLRP3 antibody. Colocalization was observed by confocal immunofluorescent microscopy. (A) Untreated cells showing the YFP-ASC expression and low level, constitutive NLRP3 expression. After exposure to either (B) LPS/ATP or (C) *G. vaginalis*, bright speck-like fluorescence was observed in the cytoplasm (arrows) indicative of colocalization of ASC with NLRP3. Images are representative of at least three replicates for each treatment.

### 3.3 NLRP3 and caspase-1 are required for *G. vaginalis* induction of IL-1β

In THP-1 cells with an integrated retrovirus encoding shRNA for the degradation of NLRP3 mRNA (Fig. 3E), we observed no differences in the TNF-α response of LPS/ATP or *G. vaginalis* treated cells (Fig. 3A), but there was a significant reduction in IL-1β release (Fig. 3B), indicating that *G. vaginalis*-induced IL-1β is dependent upon NLRP3 inflammasome recruitment. Furthermore, addition of the caspase-1 inhibitor, YVAD, was able to completely ameliorate the IL-1β response, indicating that caspase-1 dependent inflammasome recruitment is solely responsible for IL-1β secretion (Fig. 3C). This was further verified by direct analysis of caspase-1 activation using a FLICA reagent that binds with high affinity to the active form of caspase-1. This confirmed the elevation of capsase-1 activity in *G. vaginalis* treated cells and inhibition in the presence of YVAD (Fig 3D).

### 3.4 *G. vaginalis* induces NLRP3 colocalization with ASC

Because IL-1β and IL-18 secretion and pyroptotic cell death in response to *G. vaginalis* were indicative of inflammasome activation, we then investigated which inflammasome components were recruited in response to the bacterium using THP-1 cells stably expressing YFP-ASC. In untreated cells stained with anti-NLRP3 antibodies, YFP-ASC is visible, but endogenous NLRP3 is expressed at only low levels (Fig. 3A). LPS/ATP treatment increased NLRP3 intensity with NLRP3 immunoreactivity colocalizing with YFP-ASC in the same optical plane

(Fig. 3B). Treatment of cells with *G. vaginalis* also resulted in colocalization of NLRP3 staining with YFP-ASC (Fig. 3C).

## 4. DISCUSSION

*G. vaginalis* is one of the most frequently isolated bacteria in women with BV and possesses virulence traits such as sialidase, biofilm formation, and the production of a cholesterol-dependent cytolysin, vaginolysin (von Nicolai et al., 1984; Gelber et al., 2008; Swidsinski et al., 2008). Despite the more serious consequences connected with *G. vaginalis* and other BV-associated bacteria, there is scant evidence of an inflammatory response in the vaginal tracts of infected women. Rampersaud et al.( 2012) have proposed that the BV-associated outcomes may be attributed to the ascension of bacteria into the intrauterine space. However, to better understand the morbidities associated with *G. vaginalis* and other BV-associated bacteria, the role of the host cell inflammatory responses to these bacteria needs to be elucidated.

In this study we demonstrate that *G. vaginalis* induces an inflammatory response in THP-1 macrophage-like cells. Macrophages are present just beneath the epithelium in the lamina propria of the vaginal tract and have been shown to express receptors for the innate immune response (Shen et al., 2009). THP-1 cells treated with *G. vaginalis* secreted significant amounts of TNF-α, IL-1β, and IL-18. These cytokine increases indicate both the activation of NF-κB through toll-like receptors, resulting in TNF- α secretion, and inflammasome complexes,

through Nod-like receptors, resulting in IL-1 β and IL-18 secretion. Inflammasomes regulate secretion of IL-1β and IL-18 by binding and cleaving caspase-1, which subsequently cleaves and activates pro-cytokines IL-1β and IL-18 to their secreted, biologically active forms. Western blot analysis confirmed that caspase-1 cleavage and subsequent cytokine secretion occurs in *G. vaginalis* treated cells. In a study by Genc and Onderdonk (2011), colonization with *G. vaginalis* was found to be a strong indicator of preterm birth. Caspase-1 cleavage is also associated with preterm labor (Gotsch et al., 2008). After significant inflammation, cells may go through an inflammatory form of programmed cell death known as pyroptosis, leading to further inflammation in surrounding tissue. In this study, we observed that *G. vaginalis* and LPS/ATP-treated cells were both Sytox Green and Annexin V positive due to the membrane damage and loss of membrane asymmetry associated with pyroptosis. Double staining can also be indicative of late apoptotic cells as was observed for the staurosporine control. However, Western blotting for caspase-8 cleavage showed that although caspase-8 levels were upregulated in both *G. vaginalis* and LPS/ATP-treated cells, there was not significant caspase-8 cleavage in the treated THP-1 cells signifying that the cells were undergoing pyroptotic cell death. After treatment of YFP-ASC expressing THP-1 cells with *G. vaginalis*, YFP-ASC accumulated with NLRP3 in bright, punctate cytoplasmic clusters indicative of NLRP3 inflammasome formation. Caspase-1 cleavage may be engaged by other members of the Nod-like family of receptors, including

NLRC4, NLRP1, and AIM2, signaled by molecules such as *Salmonella enterica* flagellin, anthrax lethal toxin, and dsRNA, respectively. Each of these inflammatory activators regulates the release of IL-1β and IL-18 (Schroder and Tschopp 2010). Further, the reduction of NLRP3 levels reduced the secretion of IL-1β, confirming the necessity of NLRP3 in *G. vaginalis* induced inflammasome recruitment. Bacterial components, including RNA, cell wall components, and pore-forming toxins can activate NLRP3 (Schroder and Tschopp 2010). Pore-forming toxins, including listerolysin from *Listeria monocytogenes* and streptolysin O from *Streptococcus pyogenes*, have been shown to be critical for NLRP3 activation (Mariathasan et al., 2006; Harder et al., 2009). *G. vaginalis* produces the cholesterol-dependent pore-forming toxin, vaginolysin, shown to have strong homology to other pore-forming toxins (Gelber et al., 2008). The secretion of this toxin may increase membrane permeability of host cells resulting in the K+ efflux necessary for caspase-1 activation (Muñoz-Planillo et al., 2013).

In some cases, such as through the leaderless secretory pathway, IL-1β is secreted without inflammasome involvement, and is able to be cleaved by other means. In this study we confirm that IL-1β secretion in THP-1 cells is regulated by caspase-1, and that the inflammatory response is the result of an inflammasome-dependent pathway. The use of inhibitors to reduce this response is indicative of the potential therapeutic benefits to IL-1β inhibitors for the prevention of preterm birth.

The pathogenic impact of *G. vaginalis* has been the subject of debate for nearly 40 years, with observations that both implicate and absolve it in multiple roles of pathoegensis (Catlin, 1992; Swidsinski et al., 2008). The involvement of *G. vaginalis* has been questioned partly because its presence, in some studies, seems independent of disease state (Mikamo et al., 2000; Buhimschi et al., 2005). Here, we have shown that *G. vaginalis* can induce highly inflammatory conditions over a 24 h period, including pyroptotic cell death and the secretion of the proinflammatory cytokines, TNF-α and IL-1β, at levels comparable to those observed in BV (Zaga-Clavellina et al., 2012). If the bacterium ascends into the uterine environment, these inflammatory conditions in the upper genital tract could be detrimental to the amniotic membrane leading to increased permeabilization and irritation of the cervix, both of which can lead to preterm birth (Buhimschi et al., 2005). BV also significantly increases the likelihood of HIV transmission both during preganancy and postpartum, with the presence of *G. vaginalis* particularly associated with antepartum transmission (Frank et al., 2012). Inflammatory cytokines can also cause an increase in leukocyte infiltrate, providing a direct method for HIV binding to CD4 receptors and co-receptors.

This study has shown that infection of THP-1 cells with *G. vaginalis* not only results in TNF-α secretion, but also triggers the formation of the NLRP3 inflammasome leading to caspase-1 cleavage and IL-1β and IL-18 secretion and subsequent pyroptotic THP-1 cell death. The study advances the understanding that although *G. vaginalis* may not be the sole etiologic agent of BV, the innate

immune response against *G. vaginalis* is sufficient for producing the cytokines associated with BV and with the development of subsequent complications in the upper genital tract, including pelvic inflammatory disease, postpartum infections, preterm birth, and increased HIV transmission.

**ACKNOWLEDGEMENTS**

We would like to thank Mikhail Gavrilin of the Ohio State University Medical School for the generous gift of THP-1 cells expressing YFP-ASC, and Dr. Jenny P.Y. Ting for the generous gift of the NLRP3 knockdown THP-1 cells and scrambled RNA control THP-1 cells. We would additionally like to thank Dr. Elliot Altman, Ronni Altman, the Molecular Biosciences Ph.D. program and the Biology Department of MTSU, and acknowledge the support of the NSF GK-12 Grant # DGE-0841370.

## REFERENCES

Atianand, M.K., Duffy, E.B., Shah, A., Kar, S., Malik, M., Harton, J.A., 2011. *Francisella tularensis* reveals a disparity between human and mouse NLRP3 inflammasome activation. J. Biol. Chem. 286, 39033–39042.

Bergsbaken, T., Fink, S.L., Cookson, B.T., 2009. Pyroptosis: host cell death and inflammation. Nat. Rev. Microbiol. 7, 99–109.

Borchardt, K.A., Adly, B.S., Smith, R.F., Eapen, J., Beal, C.B., 1989. Importance of *Gardnerella vaginalis* as an aetiological agent in bacterial vaginosisbacterial vaginosis. Genitourin. Med. 65, 285.

Buhimschi, I.A., Christner, R., Buhimschi, C.S., 2005. Proteomic biomarker analysis of amniotic fluid for identification of intra-amniotic inflammation. BJOG Int. J. Obstet. Gynaecol. 112, 173–181.

Catlin, B.W., 1992. *Gardnerella vaginalis*: characteristics, clinical considerations, and controversies. Clin. Microbiol. Rev. 5, 213–237.

Cauci, S., Hitti, J., Noonan, C., Agnew, K., Quadrifoglio, F., Hillier, S.L., Eschenbach, D.A., 2002. Vaginal hydrolytic enzymes, immunoglobulin A against *Gardnerella vaginalis* toxin, and risk of early preterm birth among women in preterm labor with bacterial vaginosis or intermediate flora. Am. J. Obstet. Gynecol. 187, 877–881.

Costa, A., Gupta, R., Signorino, G., Malara, A., Cardile, F., Biondo, C., et al., 2012. Activation of the NLRP3 inflammasome by Group B Streptococci. J. Immunol. 188, 1953–1960.

Duncan, J.A., Gao, X., Huang, M.T.-H., O'Connor, B.P., Thomas, C.E., Willingham, S.B., et al., 2009. *Neisseria gonorrhoeae* activates the proteinase cathepsin B to mediate the signaling activities of the NLRP3 and ASC-containing Inflammasome. J. Immunol. 182, 6460–6469.

Fink, S.L., Cookson, B.T., 2006. Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. Cell. Microbiol. 8, 1812–1825.

Frank, D.N., Manigart, O., Leroy, V., Meda, N., Valéa, D., Zhang, W., et al., 2012. Altered vaginal microbiota are associated with perinatal mother-to-child transmission of HIV in African women from Burkina Faso. J. Acquir. Immune Defic. Syndr. 60, 299–306.

Gavrilin, M.A., Abdelaziz, D.H.A., Mostafa, M., Abdulrahman, B.A., Grandhi, J., Akhter, A., et al., 2012. Activation of the pyrin inflammasome by intracellular *Burkholderia cenocepacia*. J. Immunol. 188, 3469–3477.

Gelber, S.E., Aguilar, J.L., Lewis, K.L.T., Ratner, A.J., 2008. Functional and phylogenetic characterization of vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*. J. Bacteriol. 190, 3896–3903.

Genc, M.R., Onderdonk, A., 2011. Endogenous bacterial flora in pregnant women and the influence of maternal genetic variation. BJOG Int. J. Obstet. Gynaecol. 118, 154–163.

Giraldo, P.C., de Carvalho, J.B.J., do Amaral, R.L.G., da Silveira Gonçalves, A.K., Eleutério, J., Jr, Guimarães, F., 2012. Identification of immune cells

by flow cytometry in vaginal lavages from women with vulvovaginitis and normal microflora. Am. J. Reprod. Immunol. 67, 198–205.

Gotsch, F., Romero, R., Chaiworapongsa, T., Erez, O., Vaisbuch, E., Espinoza, J., et al., 2008. Evidence of the Involvement of caspase-1 under physiologic and pathologic cellular stress during human pregnancy: a link between the inflammasome and parturition. J. Matern.-Fetal Neonatal Med. 21, 605–616.

Grahames, C.B., Michel, A.D., Chessell, I.P., Humphrey, P.P., 1999. Pharmacological characterization of ATP- and LPS-induced IL-1β release in human monocytes. Br. J. Pharmacol. 127, 1915–1921.

Greten, F.R., Arkan, M.C., Bollrath, J., Hsu, L.-C., Goode, J., Miething, C., et al., 2007. NF-kappaB is a negative regulator of IL-1β secretion as revealed by genetic and pharmacological inhibition of IKKβ. Cell 130, 918–931.

Harder, J., Franchi, L., Muñoz-Planillo, R., Park, J.-H., Reimer, T., Núñez, G., 2009. Activation of the Nlrp3 inflammasome by *Streptococcus pyogenes* requires streptolysin O and NF-κB activation but proceeds independently of TLR signaling and P2X7 receptor. J. Immunol. 183, 5823–5829.

Holst, E., Wathne, B., Hovelius, B., Mårdh, P.A., 1987. Bacterial vaginosis: microbiological and clinical findings. Eur. J. Clin. Microbiol. 6, 536–541.

Li, W., Katz, B.P., Bauer, M.E., Spinola, S.M., 2013. *Haemophilus ducreyi* infection induces activation of the NLRP3 inflammasome in nonpolarized but not in polarized human macrophages. Infect. Immun. 81, 2997–3008.

Mariathasan, S., Weiss D.S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., et al., 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. Nature. 440, 228-232.

Mehta, V.B., Hart, J., Wewers, M.D., 2001. ATP-stimulated release of interleukin (IL)-1β and IL-18 requires priming by lipopolysaccharide and is independent of caspase-1 cleavage. J. Biol. Chem. 276, 3820–3826.

Mikamo, H., Sato, Y., Hayasaki, Y., Hua, Y.X., Tamaya, T., 2000. Vaginal microflora in healthy women with *Gardnerella vaginalis*. J. Infect. Chemother. 6, 173–177.

Monack, D.M., Detweiler, C.S., Falkow, S., 2001. Salmonella pathogenicity island 2-dependent macrophage death is mediated in part by the host cysteine protease caspase-1. Cell. Microbiol. 3, 825–837.

Muñoz-Planillo, R., Kuffa, P., Martinez-Colón, G., Smith, B.L., Rajendiran T.M., Nuñez, G., 2013. K+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. Immunity. 38, 1142-1153.

Park, E., Na, H.S., Song, Y.-R., Shin, S.Y., Kim, Y.-M., Chung, J., 2014. Activation of NLRP3 and AIM2 inflammasomes by *Porphyromonas gingivalis* infection. Infect. Immun. 82, 112–123.

Perregaux, D.G., McNiff, P., Laliberte, R., Conklyn, M., Gabel, C.A., 2000. ATP acts as an agonist to promote stimulus-induced secretion of IL-1β and IL-18 in human blood. J. Immunol. 165, 4615–4623.

Rampersaud, R., Randis, T.M., Ratner, A.J., 2012. Microbiota of the upper and lower genital tract. Semin. Fetal. Neonatal Med. 17, 51–57.

Santiago, G.L. dos S., Deschacht, P., El Aila, N., Kiama, T.N., Verstraelen, H., Jefferson, K.K., et al., 2011. *Gardnerella vaginalis* comprises three distinct genotypes of which only two produce sialidase. Am. J. Obstet. Gynecol. 204, 450.e1–7.

Schroder, K., Tschopp, J., 2010. The inflammasomes. Cell 140, 821–832.

Shen, R., Richter, H.E., Clements, R.H., Novak, L., Huff, K., Bimczok, D., et al., 2009. Macrophages in vaginal but not intestinal mucosa are monocyte-like and permissive to Human Immunodeficiency Virus Type 1 infection. J. Virol. 83, 3258–3267.

Strowig, T., Henao-Mejia, J., Elinav, E., Flavell, R., 2012. Inflammasomes in health and disease. Nature 481, 278–286.

Swidsinski, A., Mendling, W., Loening-Baucke, V., Swidsinski, S., Dörffel, Y., Scholze, J., et al., 2008. An adherent *Gardnerella vaginalis* biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. Am. J. Obstet. Gynecol. 198, 97.e1–6.

Turovskiy, Y., Sutyak Noll, K., Chikindas, M.L., 2011. The aetiology of bacterial vaginosis. J. Appl. Microbiol. 110, 1105–1128.

Van Oostrum, N., De Sutter, P., Meys, J., Verstraelen, H., 2013. Risks associated with bacterial vaginosis in infertility patients: a systematic review and meta-analysis. Hum. Reprod. 28, 1809–1815.

Von Nicolai, H., Hammann, R., Salehnia, S., Zilliken, F., 1984. A newly discovered sialidase from *Gardnerella vaginalis*. Zentralbl. Bakteriol. Mikrobiol. Hyg. A. 258, 20–26.

Weissenbacher, T., Walter, C., Mylonas, I., Scholz, C., Gingelmaier, A., Friese, K., 2010. Interleukin-6, interleukin-10 and interleukin-12 in vaginal fluid from women with bacterial vaginosis. Arch. Gynecol. Obstet. 281, 77–80.

Willingham, S.B., Allen, I.C., Bergstralh, D.T., Brickey, W.J., Huang, M.T.-H., Taxman, D.J., et al., 2009. NLRP3 (NALP3, cryopyrin) facilitates in vivo caspase-1 activation, necrosis, and HMGB1 release via inflammasome-dependent and -independent pathways. J. Immunol. 183, 2008–2015.

Zaga-Clavellina, V., Martha, R.V.-M., Flores-Espinosa, P., 2012. In vitro secretion profile of pro-inflammatory cytokines IL-1β, TNF-α, IL-6, and of human β-defensins (HBD)-1, HBD-2, and HBD-3 from human chorioamniotic membranes after selective stimulation with *Gardnerella vaginalis*. Am. J. Reprod. Immunol. 67, 34–43.

Zariffard, M.R., Novak, R.M., Lurain, N., Sha, B.E., Graham, P., Spear, G.T., 2005. Induction of tumor necrosis factor- alpha secretion and toll-like receptor 2 and 4 mRNA expression by genital mucosal fluids from women with BV. J. Infect. Dis. 191, 1913–1921.

# Chapter II

## *Gardnerella vaginalis* NLRP3 INFLAMMASOME FORMATION AND DIFFERENTIATION IN THP-1 MONOCYTIC CELLS IS STRAIN-DEPENDENT

Vick, E.J., Park, H.S., Huff, K.F., Brooks, K.M., Farone, A.L., Farone, M.B., n.d. *Gardnerella vaginalis* NLRP3 Inflammasome Formation and Differentiation in THP-1 Monocytic Cells is Strain-dependent. Am. J. Reprod. Immunol. In Preparation.

### 1. INTRODUCTION

It has been understood for some time that inflammation of the placenta and decidual space during pregnancy leads to pre-term birth. *Gardnerella vaginalis* is known to play a role in BV, pre-term birth, as well as other inflammatory processes, but the role of the bacterium in the context of the innate immune system is poorly explored, especially with regard to pregnancy (Catlin, 1992). Decidual macrophages and dendritic cells constitute one of the primary means of innate immune involvement during pregnancy and are responsible for the regulation of the decidual environment. Their differentiation is influenced by normal trophoblast turnover during pregnancy, and predominates with an M2 anti-inflammatory response (Abrahams et al., 2004). However, these cells express high levels of Nod-like receptors, which regulate recruitment of caspase-1, which in turn regulates the proteolytic activation and secretion of IL-1β and other pro-inflammatory cytokines. (Schroder and Tschopp, 2010). IL-1β is one of

a few central cytokines capable of triggering trophoblast apoptosis in itself, as well as larger effects such as cervical dilation (George et al., 1993; Nilkaeo and Bhuvanath, 2006).

In infections of the upper genital tract during pregnancy, the otherwise regulated turnover of trophoblasts by macrophages can cause sustained inflammation leading to extensive apoptosis of trophoblasts, and the thinning and premature rupture of the amniotic lining (Mulla et al., 2013). Macrophages continuously responding to infection release pro-inflammatory cytokines and are directed toward an M1 inflammatory phenotype (Kim et al., 2007). In this study, we explore the ability of different *G. vaginalis* strains to cause monocyte differentiation, and determine the role of NLRP3 and caspase-1 in the strain-dependent secretion of IL-1β.

## 2. MATERIALS AND METHODS

**2.1 Monocyte cell lines and bacteria.** THP-1 monocyte cells (ATCC TIB-202) were cultured in RPMI 1640 supplemented with 10% FBS (Atlanta Biologicals), 100 IU penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich) and incubated at 37°C in 5% $CO_2$. ASC-YFP THP-1 cells expressing ASC-YFP were the generous gift of Mikhail Gavrilin of the Ohio State University (Columbus, OH, USA). These THP-1 cells contain a pLenti6/V5 TOPO vector with human ASC fused to the C terminus of yellow fluorescent protein (YFP) resulting in stable expression of YFP-ASC (Gavrilin et al., 2012). The shNLRP3 and shCtrl RNA

expressing THP-1s were a generous gift of the Ting laboratory at the University of North Carolina, Chapel Hill and were used to prepare retroviral vectors and THP-1 cells stably expressing shRNA (Duncan et al., 2009). Knockdowns were verified by Western blot with anti-NLRP3 antibody (Santa Cruz Biotechnology, sc66846).

*Gardnerella vaginalis* ATCC 14018 (strain 18), 14019 (strain 19), and 49145 (strain 45) were cultured on brain heart infusion (BHI) agar (Becton Dickinson) for 48 h, then inoculated into BHI broth and grown until mid-log phase at 36 h. Bacterial cells were spun at 10,000xg for 5 min and resuspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS) before infection.

**2.2 IL-1β and caspase-1 activation.** THP-1 cells were plated at $5.0 \times 10^5$ cells/mL in twenty-four well tissue culture plates and differentiated as above. Cells were treated with either fresh pre-warmed medium, medium containing 100 ng/mL *Salmonella enterica* LPS (Sigma-Aldrich), 100 μM YVAD (Ac-YVAD-cmk, Sigma-Aldrich), medium containing *G. vaginalis* at an MOI of 5 or medium containing both YVAD and *G. vaginalis.* ATP (5mM) (Fisher Scientific) was added to LPS wells for inflammasome studies 30 min prior to harvest. Cell culture supernatants were collected at 24 h after treatment and spun at 12,000×g for 5 min at 4°C. Supernatants were collected and cytokines quantified using ELISA for IL-1β (R & D Systems) according to the manufacturer's instructions. ELISA data were analyzed using the SpectraMax M5 microplate reader and SoftMax

Pro software (Molecular Devices). For the lactate dehydrogenase assay (LDH assay, Promega) for cell death was performed according to manufacturer's instructions. Briefly, treated cell supernatants were harvested after 24 h of treatment as above, centrifuged at 12,000xg for 5 min, and then incubated according to manufacturer's instructions. Analysis was performed at 240 nm using the SpectraMax M5 microplate reader and SoftMax Pro software (Molecular Devices). Controls were included for normalization including triton-X 100 lysis controls and *G. vaginalis* only controls.

**2.3 Immunofluorescent microscopy.** THP-1 ASC-YFP expressing cells were grown on sterile coverslips at a density of $5.0 \times 10^5$ cells/mL and differentiated with 100 nM PMA (Sigma-Aldrich) before treatment. Cells were then treated with fresh pre-warmed medium, 19, or 45 at an MOI of 5. After 24 h of treatment, all media was removed, and cells were washed and fixed. Anti-NLRP3 antibodies (Santa Cruz Biotechnology) were added to the fixed cells followed by incubation at room temperature. Coverslips were washed, incubated with anti-rabbit IgG AlexaFlour 594 conjugate in antibody buffer in the dark, followed by washing and post-fixation. Stained coverslips were mounted onto glass slides using Prolong Gold antifade-reagent with DAPI (Life Technologies), and allowed to cure overnight at 4 °C. Slides were imaged using a Nikon C1 laser scanning confocal microscope and EZ-C1 acquisition software.

**2.4 Flow cytometry.** Undifferentiated THP-1 cells were plated at $5.0 \times 10^5$

cells/mL in untreated medium, with 100 ng/mL LPS, 100 nM PMA, or medium containing either *G. vaginalis* 19 or 45 at an MOI of 5 for 24 h. Medium containing THP-1 cells was removed and wells were washed with PBS then incubated for 10 min with PBS containing 5 mM EDTA to remove adherent cells. Cells were blocked in ice-cold FACS buffer (10% FBS, 0.1% sodium azide in PBS) for 10 min on ice, and then incubated with 2.5 µg/mL mouse anti-CD206 antibody (BD Pharmingen) for 30 min on ice. Cells were washed three times with cold FACS buffer then analyzed by flow cytometry.

For caspase-1 recruitment assays, cells were differentiated as before, and treated with *G. vaginalis* at an MOI of 5, DMSO, LPS/ATP, YVAD, YVAD and *G. vaginalis,* or medium alone, and incubated as above. The caspase-1 FLICA 660 kit (Immunochemistry Technologies) was used according to manufacturer's instructions to assess the degree of caspase-1 activation. Cells were removed from plates by cold 5 mM EDTA in PBS, and cells were quantified by flow cytometry as above.

**2.5 Statistical analysis.** Each experiment was performed as a minimum of three independent biological repeats, in triplicate. Data is expressed as the mean ± S.E. and analyzed either by one-way ANOVA using the Dunnett's test of multiple comparisons, or a paired student's t-test ($p < 0.05$).

## 3. RESULTS

Based on previous results with *G. vaginalis* treatment of monocytes, we sought to determine to what extent other strains of *G. vaginalis* were capable of inflammation. Strains 18 and 45 resulted in inflammasome formation (Fig 1A), caspase-1 activation (Fig 1B), and IL-1β secretion (Fig 1C), while strain 19 seemed to have little affect on the monocytes (Fig 1A-C) though the bacteria were viable and capable of replicating (data not shown). To determine if NLRP3, was the essential Nod-like receptor involved in inflammasome assembly, THP-1 NLRP3 knock-down (KD) cells were differentiated and cultured with all three strains. Both strains 18 and 45 induced significantly reduced IL-1β secretion by the reduction of NLRP3, but again strain 19 demonstrated no elevation and was not significantly affected by the reduction of NLRP3 (Fig 1D). Inhibition of caspase-1 recruitment by addition of YVAD caused reduced IL-1β secretion in strains 18 and 45 but not 19 (Fig 1C).

The response of undifferentiated THP-1 cells demonstrates the ability of *G. vaginalis* to induce differentiation of monocytes while leading to their death in the same strain-dependent manner as seen with inflammation in differentiated monocytes. Staining with CD206, a marker of mature macrophages, showed that strain 18 and 45 elicited significant differentiation which was comparable to LPS and PMA, while strain 19 was unable to promote monocyte differentiation (Fig 2A). Lactate dehydrogenase assay showed that cell death could be reduced by treatment with YVAD, showing that pyroptosis contributes to the cell death (Fig

2B). Furthermore, no significant cell death was observed in response to strain 19 and cell death in treatment with strain 45 was significantly reduced after treatment with YVAD. Treatment with YVAD appeared to reduce cell death in co-culture with strain 18, but this was not significant, implying that at least in this strain, there may be additional mechanisms involved beyond pyroptosis.

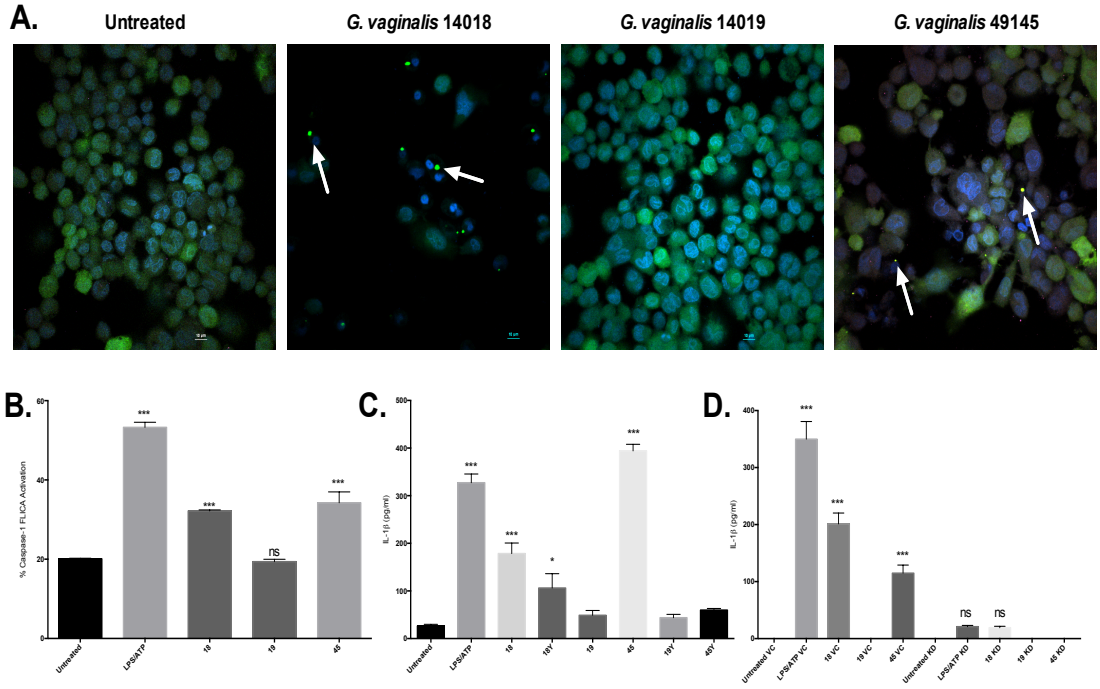



**Figure 3.1 Inflammatory effects of *Gardnerella vaginalis* strains on THP-1 macrophage-like cells is strain-dependent**

THP-1 cells stably expressing YFP-ASC were differentiated on sterile coverslips using 100 nM PMA and treated with *G. vaginalis* at an MOI of 5, 100 ng/ml LPS and 5 mM ATP 30 min prior to harvest, or fresh medium (untreated) for 24 h before fixation and staining with DAPI. (A) Untreated cells showing the YFP-ASC expression, *G. vaginalis* strain 18 showing bright speck-like fluorescence observed in the cytoplasm indicative of inflammasome activation, strain 19 showing similar characteristics to untreated control, and strain 45 showing the same speck light fluorescence indicative of inflammasome activation. Images are representative of at least three replicates for each treatment. B) THP-1 cells were treated with *G. vaginalis* at an MOI of 5, 100

ng/mL LPS and 5mM ATP 30 min prior to harvest, or medium alone (untreated). (B) Caspase-1 activation was verified by FLICA staining followed by flow cytometry, demonstrating activation of caspase-1 by strains 18 and 45. (C) The addition of the caspase-1 inhibitor Ac-YVAD-cmk (100 μM) to *G. vaginalis* treated cells, reduced IL-1β secretion in cells treated with strains 18 and 45, but had no effect on strain 19. (D) THP-1 cells stably transduced with NLRP3-specific shRNA (shNLRP3 knockdown, KD), or THP-1 cells transduced with the vector control shRNA (shCtrl, VC) were treated with *G. vaginalis* at an MOI of 5, 100 ng/mL LPS and 5mM ATP 30 min prior to harvest, or medium alone (untreated). IL-1β release was significantly reduced in knockdown THP-1 cells treated with LPS/ATP or *G. vaginalis* indicating the necessity of NLRP3 inflammasome activation in the case of strains 18 and 45. Results are the mean ± S.E. ($n \geq 3$) and are representative of at least three independent experiments. Statistical differences between groups were determined by a two-tailed t test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

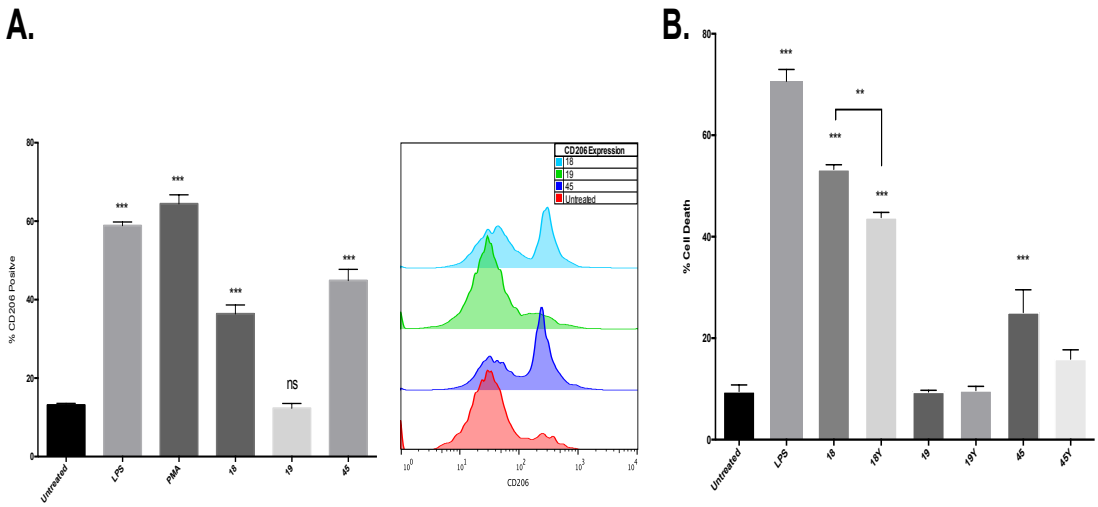



**Figure 3.2 Differentiation and cell death in THP-1 monocytes is strain-dependent**

THP-1 cells were cultured with 100 ng/mL PMA, *G. vaginalis* at an MOI of 5, 100 ng/mL LPS and 5mM ATP 30 min prior to harvest, or fresh medium (untreated) for 24h, then stained with anti-CD206 antibody, or isotype control and analyzed by flow cytometry. (A) Cells exposed to strain 18 and 45 differentiated significantly when compared to control, but were not as potent as LPS/ATP or PMA. Strain 19, as in other tests, did not generate an appreciable response. (B) THP-1 cells were cultured with *G. vaginalis* at an MOI of 5, 100 ng/mL LPS and 5mM ATP 30 min prior to harvest, 100 µM Ac-YVAD-cmk with each *G. vaginalis* strain or fresh medium (untreated). THP-1 cells were cultured for 24h and surpernatants were collected, spun at 10,000xg for 5 min to remove residual

bacteria and cell debris, then analyzed by LDH assay. *G. vaginalis* and complete lysis controls were used to obtain percentage cell death. LDH release demonstrates that cells die in response to *G. vaginalis* strains 18 and 45, but those effects are reduced by the presence of the caspase-1 inhibitor YVAD. Strain 19 shows no evidence of causing cell death. Results are the mean ± S.E. ($n \geq 3$) and are representative of at least three independent experiments. Statistical differences between groups were determined by a two-tailed t test (*$p < 0.05$, **$p < 0.01$, ***$p<0.001$).

## 4. DISCUSSION

The reason that women have different responses to *G. vaginalis* has been debated for over 40 years, largely due to the presence of *G. vaginalis* in otherwise healthy women. Many factors have been suggested as contributing to the variance observed in clinical cases, including the variability of TLR4 expression, the expression of sialidase by *G. vaginalis*, and presence of *Lactobacillus*. While all of these undoubtedly contribute to the origin of BV, this paper identifies the variance of interaction between innate immune reaction and multiple strains of *G. vaginalis.* In this study we have shown that the pro-inflammatory characteristics of *G. vaginalis*, most notably inflammasome formation, vary significantly between strains. Strain 18 and 45 were capable of stimulating differentiation, inflammasome formation, and NLRP3-dependent IL-1β secretion, and were caspase-1 dependent. In contrast, we have shown that strain 19 stimulates little if any effect in THP-1 cells in terms of inflammation or differentiation. This variance is important, especially as it relates to preterm birth, showing that *G. vaginalis* can cause the differentiation of monocytes, production of clinically important inflammatory cytokines, and cell death causing the cycle of inflammation to repeat and expand. The factors are known to contribute to preterm birth and preeclampsia. The lack of effect caused by strain 19 suggests that colonization by this strain may not lead to the same types of deleterious effects as observed other strains. This provides preliminary answers as to how

the presence of *G. vaginalis* in itself is not sufficient to indicate a potential pathology, but is dependent on the strain and likely dependent on the individual. Future studies will investigate the differences between the bacterial strains and how these differences correspond to their role in the inflammatory response to *Gardnerella vaginosis*.

## ACKNOWLEDGEMENTS

We would like to thank Mikhail Gavrilin of the Ohio State University Medical School for the generous gift of THP-1 cells expressing YFP-ASC, and Dr. Jenny P.Y. Ting for the generous gift of the NLRP3 KD THP-1 cells and scrambled RNA control THP-1 cells. We would additionally like to thank Dr. Elliot Altman, Ronni Altman, the Molecular Biosciences Ph.D. program and the Biology Department of MTSU, and acknowledge the support of the NSF GK-12 Grant # DGE-0841370.

## REFERENCES

Abrahams, V.M., Kim, Y.M., Straszewski, S.L., Romero, R., Mor, G., 2004. Macrophages and apoptotic cell clearance during pregnancy. Am. J. Reprod. Immunol. 51, 275–282.

Catlin, B.W., 1992. *Gardnerella vaginalis*: characteristics, clinical considerations, and controversies. Clin. Microbiol. Rev. 5, 213–237.

Duncan, J.A., Gao, X., Huang, M.T.-H., O'Connor, B.P., Thomas, C.E., Willingham, S.B., Bergstralh, D.T., Jarvis, G.A., Sparling, P.F., Ting, J.P.-Y., 2009. *Neisseria gonorrhoeae* activates the proteinase cathepsin B to mediate the signaling activities of the NLRP3 and ASC-Containing inflammasome. J. Immunol. 182, 6460–6469.

Gavrilin, M.A., Abdelaziz, D.H.A., Mostafa, M., Abdulrahman, B.A., Grandhi, J., Akhter, A., Khweek, A.A., Aubert, D.F., Valvano, M.A., Wewers, M.D., Amer, A.O., 2012. Activation of the pyrin inflammasome by intracellular *Burkholderia cenocepacia*. J. Immunol. 188, 3469–3477.

George, S.S., Matthews, J., Jeyaseelan, L., Seshadri, L., 1993. Cervical ripening induces labour through interleukin-1 β. Aust. N. Z. J. Obstet. Gynaecol. 33, 285–286.

Kim, J.-S., Romero, R., Cushenberry, E., Kim, Y.M., Erez, O., Nien, J.K., Yoon, B.H., Espinoza, J., Kim, C.J., 2007. Distribution of CD14+ and CD68+

macrophages in the placental bed and basal plate of women with preeclampsia and preterm labor. Placenta 28, 571–576.

Mulla, M.J., Myrtolli, K., Tadesse, S., Stanwood, N.L., Gariepy, A., Guller, S., Norwitz, E.R., Abrahams, V.M., 2013. Cutting-edge report: TLR10 plays a role in mediating bacterial peptidoglycan-induced trophoblast apoptosis. Am. J. Reprod. Immunol. 69, 449–453.

Nilkaeo, A., Bhuvanath, S., 2006. Interleukin-1 Modulation of human placental trophoblast proliferation. Mediators Inflamm. 2006, e79359.

Schroder, K., Tschopp, J., 2010. The inflammasomes. Cell 140, 821–832.

# PROJECT CONCLUSIONS

*Gardnerella vaginalis* is a commonly found resident of the human vagina and possible factor in the dysregulation of the resident microbiota. These studies characterized the inflammatory mechanisms in a human monocyte cell line in response to *G. vaginalis* that may play a role in preterm birth.

This is the first report that *G. vaginalis* stimulates the assembly of the NLRP3 inflammasome, thereby leading to the activation of caspase-1 and the secretion of pro-inflammatory cytokines IL-1β, TNF-α, and IL-18. These same cytokines are associated with the presence of the BV disease state, providing further evidence that *G. vaginalis* is involved in the development of BV. Comparing the response of THP-1 cells to the three strains of *G. vaginalis* demonstrated that there is a differential inflammasome activation and cytokine response between the three strains despite a close degree of genetic identity between the strains (Ahmed et al., 2012)

A potential role for *G. vaginalis* in preterm birth, is demonstrated by the pyroptotic cell death of human monocytes and macrophages, cells that line the decidua during pregnancy, in response to *G. vaginalis.* Although activation of apoptotic executioner caspases-3 and 7 by *G. vaginalis* was detected, this response was reduced by the addition of a caspase-1 inhibitor suggesting that the apoptotic response was a result of cell death initiated by pyroptosis (Appendix A).

Results presented in Chapter I, characterize the involvement of the Nod-like receptor NLRP3 in the inflammatory response to *G. vaginalis* strain 14018. The data showed that the majority of the inflammatory response was reduced in NLRP3 knockdown THP-1 cells, but because some IL-1β remained it was necessary to determine if NLRP3 was acting alone. Recent studies have shown the NLRP3 inflammasome can act in concert with another Nod-like protein, NLRC4 (Man et al., 2014, p. 4). NLRC4 has been associated heavily with *Salmonella spp.* especially with the release of intracellular flagellin (Zhao et al., 2011). Upon staining NLRC4 in treated THP-1 cells, we observed inflammasomes indicating that they may have a role in the initiation of inflammation as well (Appendix B). Further study is necessary to determine if these proteins are necessary for inflammasome assembly.

Characterization of three strains of *G. vaginalis* in Chapter II reveals that inflammasome activation and cytokine production in response to *G. vaginalis* are strain-dependent. This may be important in the explanation of why symptoms of *G. vaginalis* are inconsistent between studies. Of the three strains compared for these studies, consistent differences between strains 14018 and 49145 compared to strain 14019 were observed. Treatment of THP-1 cells with strain 49145 resulted in similar inflammasome activation and cytokine responses as reported for strain 14018. However, strain 14019 did not induce significant cytokine production or inflammasome activation. Additionally, strains 14018 and 49145 both caused significant differentiation and cell death of THP-1 cells,

whereas no significant differentiation or death was observed in cells treated with strain 14019. For all treatments, all strains of the organism were viable and growing following treatment of the cells. This suggests that either through a slower growth rate of strain 14019 or a difference in virulence factor expression between the strains is responsible for the reduced inflammatory response observed for strain 14019 compared to the other two strains.

Trophoblasts are cells of embryonic origin that make up the placenta and amniotic membrane. It is their role to control the integrity of the amniotic membrane, access maternal blood vessels, form chorionic villi, and further actions of homeostasis that protect the developing fetus from the maternal immune response and regulate the control of nutrients for the fetus. These cells are highly sensitive to inflammation and normally exhibit a dynamic turnover in which trophoblasts undergo natural apoptosis and are replaced by new cells while old cells are phagocytosed by decidual macrophages. Inflammation, especially from IL-1β, TNF-α, IL-6 or IL-8, disrupts the balance of this arrangement, leading to more widespread apoptosis with less cell division leading to a weakening of the amniotic membrane and an increased likelihood of chorionic rupture and cell death (Matthiesen et al., 2005). The HTR8/SVNeo first trimester trophoblast cell line was immortalized in the lab of Dr. Charles Graham, with nearly an identical expression in trophoblast genes to those removed during pregnancy (Graham et al., 1993). For this reason these cells were used as a model for trophoblast cells *in vivo*. As the interactions of *G. vaginalis* on

trophoblasts are not well understood, experiments were designed to determine whether a similar inflammatory reaction occurs in trophoblasts, and if these cells undergo a similar pyroptotic event of as was observed for THP-1 cells (Appendix C). Subsequent investigation of cell death in trophoblastic cells show that these cells that line the amniotic membrane are much less sensitive to the direct effects of *G. vaginalis.* However, it is known that proinflammatory cytokines secreted in utero lead to increased trophoblast turnover and are factors leading to placental abruption preterm birth (Thaxton et al., 2010). There was no significant secretion of IL-1β in response to exposure of trophoblast cells to any of the *G. vaginalis* strains and no caspase-1 activation was detected by flow cytometry (data not shown), indicating that either these cells respond in a different way to *G. vaginalis* than the THP-1 monocytes, or that they require much more stimulus than cells of myeloid origin, as is likely the case.

Further investigation is necessary to determine the inflammatory role of the *G. vaginalis* toxin, vaginolysin, on cell lines and *in vivo*. Additionally, with the development of a transgenic mouse line stably expressing hCD59, the receptor for vaginolysin, more accurate *in vivo* characterization of inflammation could be established. Finally, the incidence of each strain in BV is not well described. Genomic and bioinformatics techniques could go a long way in uncovering the differences between apparent inflammatory and non-inflammatory strains. Results of these studies indicate that *G. vaginalis* induces a strain-dependent

inflammatory response which can provide new context for further clinical investigation into the role of this organism in preterm birth.

## REFERENCES FROM THE INTRODUCTION AND CONCLUSIONS

Abrahams, V.M., Kim, Y.M., Straszewski, S.L., Romero, R., Mor, G., 2004. Macrophages and apoptotic cell clearance during pregnancy. Am. J. Reprod. Immunol. 51, 275–282.

Ahmed, A., Earl, J., Retchless, A., Hillier, S.L., Rabe, L.K., Cherpes, T.L., Powell, E., Janto, B., Eutsey, R., Hiller, N.L., Boissy, R., Dahlgren, M.E., Hall, B.G., Costerton, J.W., Post, J.C., Hu, F.Z., Ehrlich, G.D., 2012. Comparative genomic analyses of 17 clinical isolates of *Gardnerella vaginalis* provide evidence of multiple genetically isolated clades consistent with subspeciation into genovars. J. Bacteriol. 194, 3922–3937.

Alcivar, A., Hu, S., Tang, J., Yang, X., 2003. DEDD and DEDD2 associate with caspase-8/10 and signal cell death. Oncogene 22, 291–297.

Arlehamn, C.S.L., Evans, T.J., 2011. Pseudomonas aeruginosa pilin activates the inflammasome. Cell. Microbiol. 13, 388–401.

Atianand, M.K., Duffy, E.B., Shah, A., Kar, S., Malik, M., Harton, J.A., 2011. Francisella tularensis reveals a disparity between human and mouse NLRP3 Inflammasome activation. J. Biol. Chem. 286, 39033–39042.

Averette, K.M., Pratt, M.R., Yang, Y., Bassilian, S., Whitelegge, J.P., Loo, J.A., Muir, T.W., Bradley, K.A., 2009. Anthrax lethal toxin induced lysosomal membrane permeabilization and cytosolic cathepsin release is Nlrp1b/Nalp1b-dependent. PloS One 4, e7913.

Barlan, A.U., Griffin, T.M., McGuire, K.A., Wiethoff, C.M., 2011. Adenovirus membrane penetration activates the NLRP3 inflammasome. J. Virol. 85, 146–155.

Bedoya, F., Sandler, L.L., Harton, J.A., 2007. Pyrin-only protein 2 modulates NF-kappaB and disrupts ASC:CLR interactions. J. Immunol. Baltim. Md 1950 178, 3837–3845.

Bergsbaken, T., Fink, S.L., Cookson, B.T., 2009. Pyroptosis: host cell death and inflammation. Nat. Rev. Microbiol. 7, 99–109.

Brodsky, I.E., Medzhitov, R., 2011. Pyroptosis: macrophage suicide exposes hidden invaders. Curr. Biol. CB 21, R72–75.

Buhimschi, I.A., Christner, R., Buhimschi, C.S., 2005. Proteomic biomarker analysis of amniotic fluid for identification of intra-amniotic inflammation. BJOG Int. J. Obstet. Gynaecol. 112, 173–181.

Catlin, B.W., 1992. Gardnerella vaginalis: characteristics, clinical considerations, and controversies. Clin. Microbiol. Rev. 5, 213–237.

Cauci, S., Driussi, S., Guaschino, S., Isola, M., Quadrifoglio, F., 2002. Correlation of local interleukin-1β levels with specific IgA response against *Gardnerella vaginalis* cytolysin in women with bacterial vaginosis. Am. J. Reprod. Immunol. 47, 257–264.

Choubey, D., 2012. DNA-responsive inflammasomes and their regulators in autoimmunity. Clin. Immunol. 142, 223–231.

Chu, Z.L., Pio, F., Xie, Z., Welsh, K., Krajewska, M., Krajewski, S., Godzik, A., Reed, J.C., 2001. A novel enhancer of the Apaf1 apoptosome involved in cytochrome c-dependent caspase activation and apoptosis. J. Biol. Chem. 276, 9239–9245.

Costa, A., Gupta, R., Signorino, G., Malara, A., Cardile, F., Biondo, C., Midiri, A., Galbo, R., Trieu-Cuot, P., Papasergi, S., Teti, G., Henneke, P., Mancuso, G., Golenbock, D.T., Beninati, C., 2012. Activation of the NLRP3 inflammasome by group B *Streptococci*. J. Immunol. 188, 1953–1960.

Craven, R.R., Gao, X., Allen, I.C., Gris, D., Bubeck Wardenburg, J., McElvania-Tekippe, E., Ting, J.P., Duncan, J.A., 2009. Staphylococcus aureus alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. PloS One 4, e7446.

Criswell, B.S., Stenback, W.A., Black, S.H., Gardner, H.L., 1972. Fine structure of Haemophilus vaginalis. J. Bacteriol. 109, 930–932.

Dostert, C., Pétrilli, V., Van Bruggen, R., Steele, C., Mossman, B.T., Tschopp, J., 2008. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science 320, 674–677.

Dowds, T.A., Masumoto, J., Zhu, L., Inohara, N., Núñez, G., 2004. Cryopyrin-induced interleukin-1β secretion in monocytic cells: enhanced activity of disease-associated mutants and requirement for ASC. J. Biol. Chem. 279, 21924–21928.

Duncan, J.A., Gao, X., Huang, M.T.-H., O'Connor, B.P., Thomas, C.E., Willingham, S.B., Bergstralh, D.T., Jarvis, G.A., Sparling, P.F., Ting, J.P.-Y., 2009. Neisseria gonorrhoeae activates the proteinase Cathepsin B to mediate the signaling activities of the NLRP3 and ASC-Containing inflammasome. J. Immunol. 182, 6460–6469.

Fairbrother, W.J., Gordon, N.C., Humke, E.W., O'Rourke, K.M., Starovasnik, M.A., Yin, J.P., Dixit, V.M., 2001. The PYRIN domain: a member of the death domain-fold superfamily. Protein Sci. Publ. Protein Soc. 10, 1911–1918.

Fantuzzi, G., Dinarello, C.A., 1999. Interleukin-18 and interleukin-1β: two cytokine substrates for ICE (caspase-1). J. Clin. Immunol. 19, 1–11.

Fink, S.L., Cookson, B.T., 2006. Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. Cell. Microbiol. 8, 1812–1825.

Franchi, L., Kanneganti, T.-D., Dubyak, G.R., Núñez, G., 2007. Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria. J. Biol. Chem. 282, 18810–18818.

Frank, D.N., Manigart, O., Leroy, V., Meda, N., Valéa, D., Zhang, W., Dabis, F., Pace, N.R., Van de Perre, P., Janoff, E.N., 2012. Altered vaginal microbiota are associated with perinatal mother-to-child transmission of

HIV in African women from Burkina Faso. J. Acquir. Immune Defic. Syndr. 60, 299–306.

Gardner, H.L., Dukes, C.D., 1955. Haemophilus vaginalis vaginitis: a newly defined specific infection previously classified non-specific vaginitis. Am. J. Obstet. Gynecol. 69, 962–976.

Gavrilin, M.A., Abdelaziz, D.H.A., Mostafa, M., Abdulrahman, B.A., Grandhi, J., Akhter, A., Khweek, A.A., Aubert, D.F., Valvano, M.A., Wewers, M.D., Amer, A.O., 2012. Activation of the Pyrin inflammasome by intracellular Burkholderia cenocepacia. J. Immunol. 188, 3469–3477.

Gelber, S.E., Aguilar, J.L., Lewis, K.L.T., Ratner, A.J., 2008. Functional and phylogenetic characterization of Vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*. J. Bacteriol. 190, 3896–3903.

Genc, M.R., Onderdonk, A., 2011. Endogenous bacterial flora in pregnant women and the influence of maternal genetic variation. BJOG Int. J. Obstet. Gynaecol. 118, 154–163.

George, S.S., Matthews, J., Jeyaseelan, L., Seshadri, L., 1993. Cervical ripening induces labour through interleukin-1β. Aust. N. Z. J. Obstet. Gynaecol. 33, 285–286.

Gotsch, F., Romero, R., Chaiworapongsa, T., Erez, O., Vaisbuch, E., Espinoza, J., Kusanovic, J.P., Mittal, P., Mazaki-Tovi, S., Kim, C.J., Kim, J.S., Edwin, S.S., Nhan-Chang, C.-L., Hamill, N., Friel, L.A., Than, N.G., Mazor, M., Yoon, B.H., Hassan, S.S., 2008. Evidence of the involvement of Caspase-

1 under physiologic and pathologic cellular stress during human pregnancy: A link between the inflammasome and parturition. J. Matern.-Fetal Neonatal Med. 21, 605–616.

Grahames, C.B., Michel, A.D., Chessell, I.P., Humphrey, P.P., 1999. Pharmacological characterization of ATP- and LPS-induced IL-1β release in human monocytes. Br. J. Pharmacol. 127, 1915–1921.

Greenwood, J.R., 1983. Current taxonomic status of Gardnerella vaginalis. Scand. J. Infect. Dis. Suppl. 40, 11–14.

Gross, O., Thomas, C.J., Guarda, G., Tschopp, J., 2011. The inflammasome: an integrated view. Immunol. Rev. 243, 136–151.

Guo, H., Gao, J., Taxman, D.J., Ting, J.P.Y., Su, L., 2014. HIV-1 infection induces interleukin-1β production via TLR8 protein-dependent and NLRP3 inflammasome mechanisms in human monocytes. J. Biol. Chem. 289, 21716–21726.

Harder, J., Franchi, L., Muñoz-Planillo, R., Park, J.-H., Reimer, T., Núñez, G., 2009. Activation of the NLRP3 inflammasome by *Streptococcus pyogenes* requires Streptolysin-O and NF-κB activation but proceeds independently of TLR signaling and P2X7 receptor. J. Immunol. 183, 5823–5829.

Hasegawa, M., Imamura, R., Kinoshita, T., Matsumoto, N., Masumoto, J., Inohara, N., Suda, T., 2005. ASC-mediated NF-kappaB activation leading to interleukin-8 production requires caspase-8 and is inhibited by CLARP. J. Biol. Chem. 280, 15122–15130.

Hiller, S., Kohl, A., Fiorito, F., Herrmann, T., Wider, G., Tschopp, J., Grütter, M.G., Wüthrich, K., 2003. NMR structure of the apoptosis- and inflammation-related NALP1 pyrin domain. Struct. 11, 1199–1205.

Hise, A.G., Tomalka, J., Ganesan, S., Patel, K., Hall, B.A., Brown, G.D., Fitzgerald, K.A., 2009. An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen *Candida albicans*. Cell Host Microbe 5, 487–497.

Hornung, V., Ablasser, A., Charrel-Dennis, M., Bauernfeind, F., Horvath, G., Caffrey, D.R., Latz, E., Fitzgerald, K.A., 2009. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature 458, 514–518.

Johnson, S., Brooks, N.J., Smith, R.A.G., Lea, S.M., Bubeck, D., 2013. Structural basis for recognition of the pore-forming toxin intermedilysin by human complement receptor CD59. Cell Rep. 3, 1369–1377.

Joosten, L.A.B., Heinhuis, B., Abdollahi-Roodsaz, S., Ferwerda, G., Lebourhis, L., Philpott, D.J., Nahori, M.-A., Popa, C., Morre, S.A., van der Meer, J.W.M., Girardin, S.E., Netea, M.G., van den Berg, W.B., 2008. Differential function of the NACHT-LRR (NLR) members Nod1 and Nod2 in arthritis. Proc. Natl. Acad. Sci. 105, 9017–9022.

Josey, W.E., Schwebke, J.R., 2008. The polymicrobial hypothesis of BV causation: a reassessment. Int. J. STD AIDS 19, 152–154.

Kambe, N., Nakamura, Y., Saito, M., Nishikomori, R., 2010. The inflammasome, an innate immunity guardian, participates in skin urticarial reactions and contact hypersensitivity. Allergol. Int. Off. J. Jpn. Soc. Allergol. 59, 105–113.

Kim, J.-S., Romero, R., Cushenberry, E., Kim, Y.M., Erez, O., Nien, J.K., Yoon, B.H., Espinoza, J., Kim, C.J., 2007. Distribution of CD14+ and CD68+ macrophages in the placental bed and basal plate of women with preeclampsia and preterm labor. Placenta 28, 571–576.

Koonin, E.V., Aravind, L., 2000. The NACHT family - a new group of predicted NTPases implicated in apoptosis and MHC transcription activation. Trends Biochem. Sci. 25, 223–224.

Koumans, E.H., Sternberg, M., Bruce, C., McQuillan, G., Kendrick, J., Sutton, M., Markowitz, L.E., 2007. The prevalence of BV in the United States, 2001-2004; associations with symptoms, sexual behaviors, and reproductive health. Sex. Transm. Dis. 34, 864–869.

Lagace-Wiens, P.R.S., Ng, B., Reimer, A., Burdz, T., Wiebe, D., Bernard, K., 2008. *Gardnerella vaginalis* bacteremia in a previously healthy man: Case report and characterization of the isolate. J. Clin. Microbiol. 46, 804–806.

Lage, S.L., Longo, C., Branco, L.M., da Costa, T.B., Buzzo, C. de L., Bortoluci, K.R., 2014. Emerging concepts about NAIP/NLRC4 inflammasomes. Front. Immunol. 5, 309.

Lamkanfi, M., Kanneganti, T.-D., 2010. NLRP3: an immune sensor of cellular stress and infection. Int. J. Biochem. Cell Biol. 42, 792–795.

Leopold, S., 1953. Heretofore undescribed organism isolated from the genitourinary system. U. S. Armed Forces Med. J. 4, 263–266.

Li, W., Katz, B.P., Bauer, M.E., Spinola, S.M., 2013. *Haemophilus ducreyi* infection Induces activation of the NLRP3 Inflammasome in nonpolarized but not in polarized human macrophages. Infect. Immun. 81, 2997–3008.

Luong, M.-L., Libman, M., Dahhou, M., Chen, M.F., Kahn, S.R., Goulet, L., Séguin, L., Lydon, J., McNamara, H., Platt, R.W., Kramer, M.S., 2010. Vaginal douching, bacterial vaginosis, and spontaneous preterm birth. J. Obstet. Gynaecol. Can. JOGC J. Obstétrique Gynécologie Can. JOGC 32, 313–320.

Man, S.M., Hopkins, L.J., Nugent, E., Cox, S., Glück, I.M., Tourlomousis, P., Wright, J.A., Cicuta, P., Monie, T.P., Bryant, C.E., 2014. Inflammasome activation causes dual recruitment of NLRC4 and NLRP3 to the same macromolecular complex. Proc. Natl. Acad. Sci. 111, 7403–7408.

Mariathasan, S., Newton, K., Monack, D.M., Vucic, D., French, D.M., Lee, W.P., Roose-Girma, M., Erickson, S., Dixit, V.M., 2004. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. Nature 430, 213–218.

Martinon, F., Pétrilli, V., Mayor, A., Tardivel, A., Tschopp, J., 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature. 440, 237–241.

Martinon, F., Tschopp, J., 2007. Inflammatory caspases and inflammasomes: master switches of inflammation. Cell Death Differ. 14, 10–22.

Mehta, V.B., Hart, J., Wewers, M.D., 2001. ATP-stimulated release of interleukin (IL)-1β and IL-18 requires priming by lipopolysaccharide and is independent of caspase-1 cleavage. J. Biol. Chem. 276, 3820–3826.

Miao, E.A., Rajan, J.V., Aderem, A., 2011. Caspase-1-induced pyroptotic cell death. Immunol. Rev. 243, 206–214.

Mikamo, H., Sato, Y., Hayasaki, Y., Hua, Y.X., Tamaya, T., 2000. Vaginal microflora in healthy women with *Gardnerella vaginalis*. J. Infect. Chemother. Off. J. Jpn. Soc. Chemother. 6, 173–177.

Mishra, B.B., Moura-Alves, P., Sonawane, A., Hacohen, N., Griffiths, G., Moita, L.F., Anes, E., 2010. *Mycobacterium tuberculosis* protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. Cell. Microbiol. 12, 1046–1063.

Monack, D.M., Detweiler, C.S., Falkow, S., 2001. *Salmonella* pathogenicity island 2-dependent macrophage death is mediated in part by the host cysteine protease caspase-1. Cell. Microbiol. 3, 825–837.

Moreira Mascarenhas, R.E., Sacramento Cunha Machado, M., Borges da Costa e Silva, B.F., Fernandes Weyll Pimentel, R., Teixeira Ferreira, T., Silva

Leoni, F.M., Grassi, M.F.R., 2012. Prevalence and risk factors for bacterial vaginosis and other vulvovaginitis in a population of sexually active adolescents from Salvador, Bahia, Brazil. Infect. Dis. Obstet. Gynecol. e378640

Mulla, M.J., Myrtolli, K., Tadesse, S., Stanwood, N.L., Gariepy, A., Guller, S., Norwitz, E.R., Abrahams, V.M., 2013. Cutting-edge report: TLR10 plays a role in mediating bacterial peptidoglycan-induced trophoblast apoptosis. Am. J. Reprod. Immunol. 69, 449–453.

Nelson, D.B., Ness, R.B., Peipert, J.F., Soper, D.E., Amortegui, A.J., Gluck, J., Wiesenfeld, H., Rice, P.A., 1998. Factors predicting upper genital tract inflammation among women with lower genital tract infection. J. Womens Health. 7, 1033–1040.

Nilkaeo, A., Bhuvanath, S., 2006. Interleukin-1 modulation of human placental trophoblast proliferation. Mediators Inflamm. e79359.

Olinger, G.G., Hashemi, F.B., Sha, B.E., Spear, G.T., 1999. Association of indicators of bacterial vaginosis with a female genital tract factor that induces expression of HIV-1. AIDS 13, 1905–1912.

Onderdonk, A.B., Lee, M.-L., Lieberman, E., Delaney, M.L., Tuomala, R.E., 2003. Quantitative microbiologic models for preterm delivery. J. Clin. Microbiol. 41, 1073–1079.

Park, E., Na, H.S., Song, Y.-R., Shin, S.Y., Kim, Y.-M., Chung, J., 2014. Activation of NLRP3 and AIM2 inflammasomes by *Porphyromonas gingivalis* infection. Infect. Immun. 82, 112–123.

Pedraza-Avilés, A.G., Zaragoza, M.C., Mota-Vázquez, R., Hernández-Soto, C., Ramírez-Santana, M., Terrazas-Maldonado, M.L., 2001. Treatment of urinary tract infection by Gardnerella vaginalis: a comparison of oral metronidazole versus ampicillin. Rev. Latinoam. Microbiol. 43, 65–69.

Peipert, J.F., Lapane, K.L., Allsworth, J.E., Redding, C.A., Blume, J.D., Stein, M.D., 2008. Bacterial vaginosis, race, and sexually transmitted infections: does race modify the association? Sex. Transm. Dis. 35, 363–367.

Perregaux, D.G., McNiff, P., Laliberte, R., Conklyn, M., Gabel, C.A., 2000. ATP acts as an agonist to promote stimulus-induced secretion of IL-1β and IL-18 in human blood. J. Immunol. 165, 4615–4623.

Pétrilli, V., Papin, S., Dostert, C., Mayor, A., Martinon, F., Tschopp, J., 2007. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. Cell Death Differ. 14, 1583–1589.

Piot, P., van Dyck, E., Goodfellow, M., Falkow, S., 1980. A taxonomic study of *Gardnerella vaginalis* (*Haemophilus vaginalis*) Gardner and Dukes 1955. J. Gen. Microbiol. 119, 373–396.

Rampersaud, R., Randis, T.M., Ratner, A.J., 2012. Microbiota of the upper and lower genital tract. Semin. Fetal. Neonatal Med. 17, 51–57.

Rottini, G., Dobrina, A., Forgiarini, O., Nardon, E., Amirante, G.A., Patriarca, P., 1990. Identification and partial characterization of a cytolytic toxin produced by *Gardnerella vaginalis*. Infect. Immun. 58, 3751–3758.

Sadhu, K., Domingue, P.A., Chow, A.W., Nelligan, J., Cheng, N., Costerton, J.W., 1989. *Gardnerella vaginalis* has a gram-positive cell-wall ultrastructure and lacks classical cell-wall lipopolysaccharide. J. Med. Microbiol. 29, 229–235.

Schroder, K., Tschopp, J., 2010. The inflammasomes. Cell 140, 821–832.

Sharma, H., Tal, R., Clark, N.A., Segars, J.H., 2014. Microbiota and pelvic inflammatory disease. Semin. Reprod. Med. 32, 43–49.

Shen, R., Richter, H.E., Clements, R.H., Novak, L., Huff, K., Bimczok, D., Sankaran-Walters, S., Dandekar, S., Clapham, P.R., Smythies, L.E., Smith, P.D., 2009. Macrophages in vaginal but not intestinal mucosa are monocyte-like and permissive to Human Immunodeficiency Virus Type 1 infection. J. Virol. 83, 3258–3267.

Skarin, A., Sylwan, J., 1986. Vaginal lactobacilli inhibiting growth of *Gardnerella vaginalis*, *Mobiluncus* and other bacterial species cultured from vaginal content of women with bacterial vaginosis. Acta Pathol. Microbiol. Immunol. Scand. [B] 94, 399–403.

Sokolovska, A., Becker, C.E., Ip, W.K.E., Rathinam, V.A.K., Brudner, M., Paquette, N., Tanne, A., Vanaja, S.K., Moore, K.J., Fitzgerald, K.A., Lacy-Hulbert, A., Stuart, L.M., 2013. Activation of caspase-1 by the NLRP3

inflammasome regulates the NADPH oxidase NOX2 to control phagosome function. Nat. Immunol. 14, 543–553.

Strowig, T., Henao-Mejia, J., Elinav, E., Flavell, R., 2012. Inflammasomes in health and disease. Nature 481, 278–286.

Swidsinski, A., Doerffel, Y., Loening-Baucke, V., Swidsinski, S., Verstraelen, H., Vaneechoutte, M., Lemm, V., Schilling, J., Mendling, W., 2010. *Gardnerella* biofilm involves females and males and is transmitted sexually. Gynecol. Obstet. Invest. 70, 256–263.

Swidsinski, A., Mendling, W., Loening-Baucke, V., Swidsinski, S., Dörffel, Y., Scholze, J., Lochs, H., Verstraelen, H., 2008. An adherent *Gardnerella vaginalis* biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. Am. J. Obstet. Gynecol. 198, 97.e1–6.

Swidsinski, A., Verstraelen, H., Loening-Baucke, V., Swidsinski, S., Mendling, W., Halwani, Z., 2013. Presence of a polymicrobial endometrial biofilm in patients with bacterial vaginosis. PloS One 8, e53997.

Thaxton, J.E., Nevers, T.A., Sharma, S., 2010. TLR-Mediated preterm birth in response to pathogenic agents. Infect. Dis. Obstet. Gynecol. e378472.

Turovskiy, Y., Ludescher, R.D., Aroutcheva, A.A., Faro, S., Chikindas, M.L., 2009. Lactocin 160, a bacteriocin produced by vaginal *Lactobacillus rhamnosus*, targets cytoplasmic membranes of the vaginal pathogen, *Gardnerella vaginalis*. Probiotics Antimicrob. Proteins 1, 67–74.

Turovskiy, Y., Sutyak Noll, K., Chikindas, M.L., 2011. The aetiology of bacterial vaginosis. J. Appl. Microbiol. 110, 1105–1128.

Virecoulon, F., Wallet, F., Fruchart-Flamenbaum, A., Rigot, J.-M., Peers, M.-C., Mitchell, V., Courcol, R.J., 2005. Bacterial flora of the low male genital tract in patients consulting for infertility. Andrologia 37, 160–165.

Von Nicolai, H., Hammann, R., Salehnia, S., Zilliken, F., 1984. A newly discovered sialidase from *Gardnerella vaginalis*. Zentralblatt Für Bakteriol. Mikrobiol. Hyg. 258, 20–26.

Willingham, S.B., Allen, I.C., Bergstralh, D.T., Brickey, W.J., Huang, M.T.-H., Taxman, D.J., Duncan, J.A., Ting, J.P.-Y., 2009. NLRP3 (NALP3, Cryopyrin) facilitates *in vivo* caspase-1 activation, necrosis, and HMGB1 Release via inflammasome-dependent and -independent pathways. J. Immunol. 183, 2008–2015.

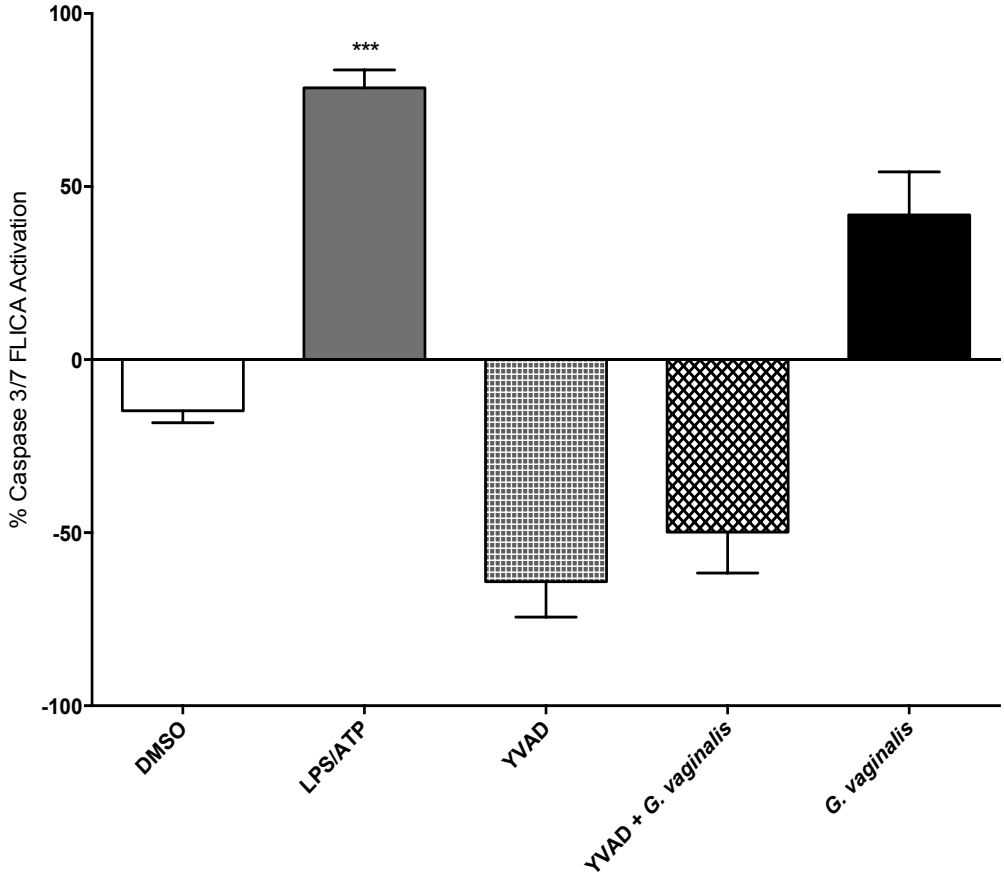
Zaga-Clavellina, V., Martha, R.V.-M., Flores-Espinosa, P., 2012. *In vitro* secretion profile of pro-inflammatory cytokines IL-1β, TNF-α, IL-6, and of human β-defensins (HBD)-1, HBD-2, and HBD-3 from human chorioamniotic membranes after selective stimulation with *Gardnerella vaginalis*. Am. J. Reprod. Immunol. 67, 34–43.

Zou, H., Yang, R., Hao, J., Wang, J., Sun, C., Fesik, S.W., Wu, J.C., Tomaselli, K.J., Armstrong, R.C., 2003. Regulation of the Apaf-1/caspase-9 apoptosome by caspase-3 and XIAP. J. Biol. Chem. 278, 8091–8098.

Zozaya-Hinchliffe, M., Lillis, R., Martin, D.H., Ferris, M.J., 2010. Quantitative PCR assessments of bacterial species in women with and without bacterial vaginosis. J. Clin. Microbiol. 48, 1812–1819.

# APPENDICES

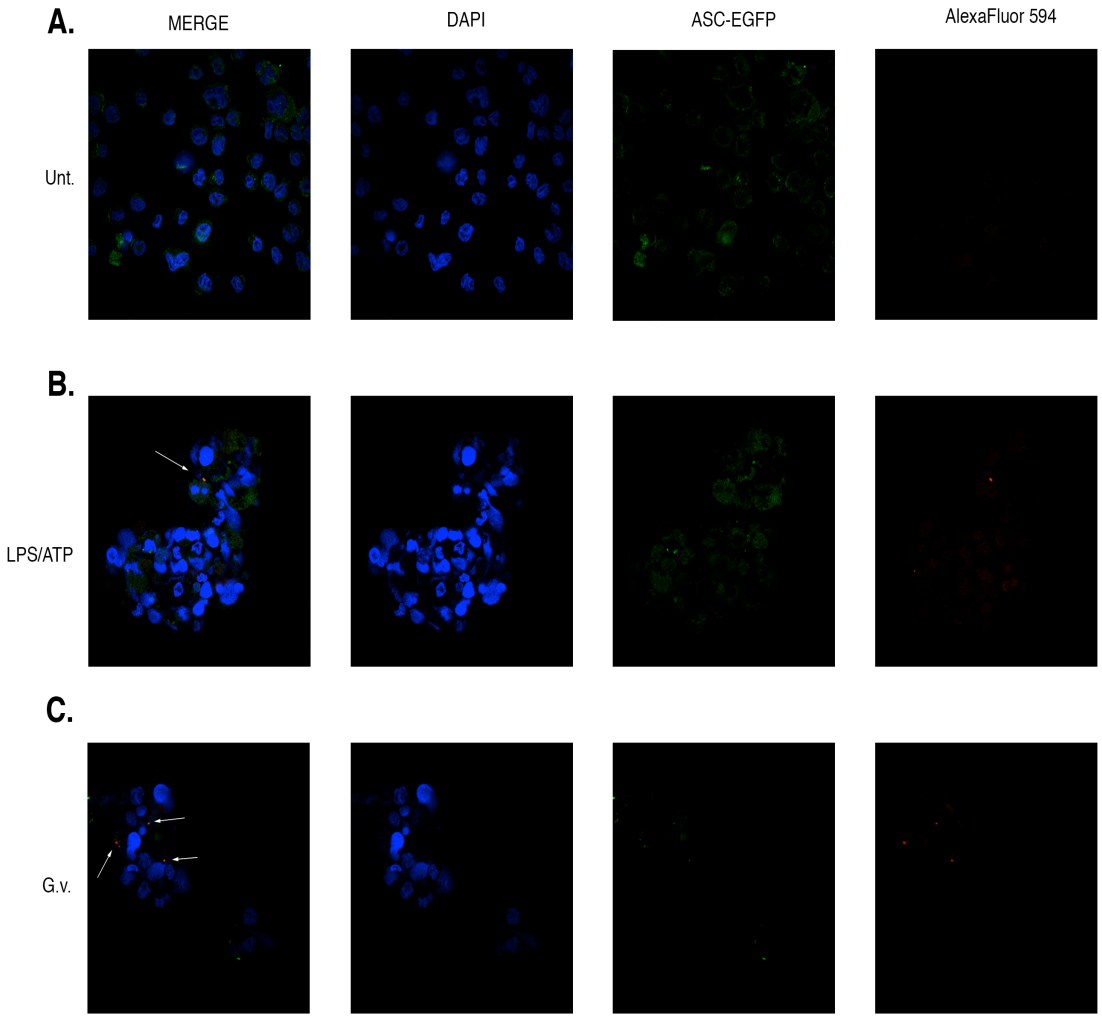
# APPENDIX A: CASPASE 3/7 ACTIVATION IN RESPONSE TO *GARDNERELLA VAGINALIS* INFECTION




**Appendix A. Apoptosis inducing caspase activation by *G. vaginalis* 14018:** THP-1 monocyte cells (ATCC TIB-202) were cultured in RPMI 1640 supplemented with 10% FBS (Atlanta Biologicals), 100 IU penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich) and incubated at 37°C in 5% $CO_2$. *Gardnerella vaginalis* ATCC 14018 was cultured on brain heart infusion (BHI) agar (Becton Dickinson) for 48 h, then inoculated into BHI broth and grown until

mid-log phase at 36 h. Bacterial cells were spun at 10,000 *xg* for 5 min and resuspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS). For caspase-3/7 recruitment assays, cells were differentiated as before, and treated with *G. vaginalis* at an MOI of 5, DMSO, LPS/ATP, YVAD, YVAD and *G. vaginalis,* or medium alone, and incubated as above. The caspase-3/7 FLICA 660 kit (Immunochemistry Technologies) was used according to manufacturer's instructions to assess the degree of caspase-3 and 7 activation. Cells were removed from plates by cold 5 mM EDTA in PBS, and cells were quantified by flow cytometry using the Guava 8HT system (Merck) and FlowJo analysis software (Treestar). Caspases 3/7 are recruited in response to *G. vaginalis*. The caspase-1 inhibitor, YVAD, significantly reduced this response, suggesting that this may be a secondary response to inflammasome activation. Results are the mean ± S.E. ($n \geq 3$) and are representative of at least three independent experiments. Statistical differences between groups were determined by a two-tailed t test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

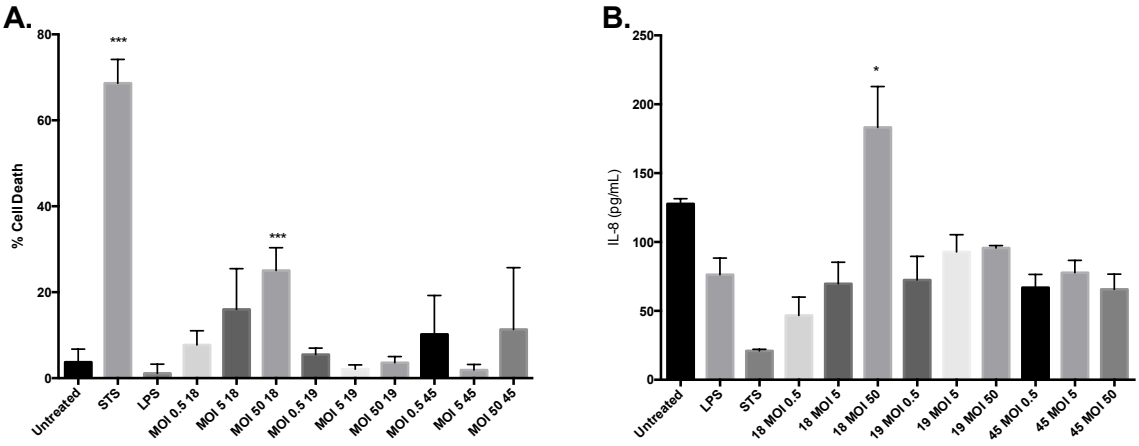
# APPENDIX B: RECRUITMENT OF THE NLRC4 INFLAMMASOME IN RESPONSE TO TREATMENT WITH *GARDNERELLA VAGINALIS* IN THP-1 CELLS




**Appendix B. NLRC4 colocalization with YFP-ASC.** THP-1 cells stably expressing YFP-ASC were grown on sterile coverslips and treated with *G. vaginalis* at a MOI of 5-10, 1 µg/ml LPS/5 mM ATP, or fresh medium (untreated) for 12 h before fixation and immunofluorescent staining with anti-NLRC4

antibody. Colocalization of YFP-ASC with NLRC4 was observed by confocal immunofluorescent microscopy. (A) Untreated cells showing the YFP-ASC expression and low level, constitutive NLRC4 expression. After exposure to either (B) LPS/ATP or (C) *G. vaginalis*, bright speck-like fluorescence was observed in the cytoplasm (arrows) indicative of colocalization of ASC with NLRC4. Images are representative of at least three replicates for each treatment.

# APPENDIX C: CELL DEATH AND IL-8 SECRETION IN RESPONSE TO *GARDNERELLA VAGINALIS* INFECTION




**Appendix C. Cell death and inflammation of HTR8 trophoblasts exposed to *G. vaginalis***

HTR8/SVneo (HTR8) cells were the generous gift of Charles Graham of Queen's University (Toronto, ON, CA). Briefly, these cells were created by stable transduction with the Large T-antigen of Simian Virus 40 (Graham et al., 1993). HTR8 cells were cultured in RPMI 1640 supplemented with 5% FBS (Atlanta Biologicals), 100 IU penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich) and incubated at 37°C in 5% $CO_2$.

*Gardnerella vaginalis* ATCC 14018, 14019 and 49145 were cultured on brain heart infusion (BHI) agar (Becton Dickinson) for 48 h, then inoculated into BHI broth and grown until mid-log phase at 36 h. Bacterial cells were spun at 10,000 xg for 5 min and resuspended in RPMI 1640 medium containing 5% FBS. HTR8

cells were plated at $1.0 \times 10^5$ cells/mL in 24-well tissue culture plates and allowed to adhere for 24 h. Cells were treated with either fresh pre-warmed medium, medium containing 100 ng/mL *Salmonella enterica* LPS (Sigma-Aldrich), 1 μM staurosporine (STS, Sigma-Aldrich), or medium containing *G. vaginalis* strain 14018, 14019, and 49145 at an MOI of 0.5, 5, or 50. ELISA data was analyzed using the SpectraMax M5 microplate reader and SoftMax Pro software (Molecular Devices). For the Lactate Dehydrogenase Assay (LDH assay, Promega) for cell death was performed according to manufacturer's instructions. Briefly, treated cell supernatants were harvested after 24 h of treatment as above, centrifuged at 12,000xg for 5 min, and then incubated according to manufacturer instructions. Analysis was performed at 240nm using the SpectraMax M5 microplate reader and SoftMax Pro software (Molecular Devices). Controls were included for normalization including triton-X 100 lysis controls and *G. vaginalis* only controls. HTR8 cells exposed to *G. vaginalis* strains 18,19, and 45 show both a dose and strain-dependent effect to strain 18. (A) Strain 18 generated the largest degree of cell death, which increased in a dose dependent fashion. Strain 19 did not generate a significant level of cell death at any dose test, and strain 45 generated variable levels of cell death and inflammation which when averaged were not significant.(B) IL-8 was produced in response to all three strains but the largest peak was again generated by strain 18. Results are the mean ± S.E. ($n \geq 3$) and are representative of at least three

independent experiments. Statistical differences between groups were determined by a two-tailed t test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).